Hydrolytic degradation of different infiltrant compositions within different histological zones of enamel caries-like-lesions

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This study evaluates the effects of resin infiltrant composition and caries histological zone on the hydrolytic degradation of resin-infiltrated artificial bovine enamel caries (RI-AEC). Different resin infiltrants were tested (n=26 per group): Icon (G1); TEGDMA 60%, UDMA 20%, and HEMA 20% (TUH); TEGDMA 80% and HEMA 20% (TH); and TEGDMA 75% and Bis-EMA 25% (TB). Following caries infiltration, samples were cut perpendicularly, and transverse microhardness were analyzed (at two histological zones: surface layer and lesion body) before and after 21 days of water immersion. TB presented lower decrease in microhardness (due to hydrolytic degradation) than the other groups, with a large effect size (Hedge’s G from 0.83 to 1.19) and high power (84 to 99%). Neither histological zone nor its interaction with resin infiltrant composition significantly affected the outcome. In conclusion, resin composition affected microhardness of RI-AEC upon water immersion, and TB was the least affected.

Keywords: Infiltrant, Resin monomers, Enamel caries, Histopathology

INTRODUCTION

The main non-operative treatment options for noncavitated enamel caries lesion are remineralization, sealant application, and resin infiltration11. Resin infiltration is performed using a resinous material that has a penetration coefficient equal to or greater than 50 cm/s, and it is aimed at occluding, as far as possible, the pores of carious enamel32.

Elimination of air from the carious enamel pores located deep in the lesion (deep in the body of the lesion) is a big challenge, making it difficult complete occlusion of carious enamel pores3,6. This is because as a liquid infiltrates into dried porous materials, the inward liquid displacement competes with the outward displacement of air, and the air in the bottom part of the solid material is the last one to be replaced5. Actually, three-dimensional analysis has shown that the resin infiltrated area in both active and inactive natural enamel caries lesion is actually a sum of occluded and non-occluded pores, and most of the non-occluded pores have been found deep in the body of the lesion8. Further quantitative volumetric data indicate that the pores of resin-infiltrated enamel caries, from the surface layer to the bottom of the body of the lesion, are filled with a combination of infiltrant, organic matter, firmly bound water, and air7,8. Thus, in resin-infiltrated enamel caries lesions, resin infiltrant interacts with water at the both the surface (contacted with the external environment) and in the pores within the enamel caries lesion.

Resin infiltrants composition influences directly its properties9,10. The addition of solvents and monomers can alter properties such as degree of conversion, hardness, elastic modulus, softening ratio, and caries penetration depth10. Both hydrophobic and hydrophilic can be found in resin infiltrants10. Resins undergo hydrolytic degradation in contact with water11, what might contribute to the decrease of the amount of resin-occluded pores overtime with resin infiltrants rich in hydrophilic monomers. The decrease in the resin-occluded pores is expected to reduce both the caries preventive and the esthetic (reduced opacity of carious enamel) outcomes.

The higher amount of air in the bottom of resin-infiltrated enamel caries lesion might contribute to a greater surface area of contact between resin infiltrant and firmly bound water, favoring hydrolytic degradation, but this hypothesis has not been tested yet.

Considering that both the histological zone of enamel caries and resin infiltrant composition might affect hydrolytic degradation of resin infiltrant, the aim of this study was to evaluate the effects of resin infiltrant composition and caries histological zone on the variation in microhardness of resin-infiltrated artificial bovine enamel caries (RI-AEC) upon water immersion hydrolytic.
MATERIALS AND METHODS

Study design and hypotheses
This was a quantitative, in vitro, transversal, experimental study with the outcome as the difference between microhardness values measured at two time intervals (0 and 21 days). It tested the effects of two factors (resin infiltrant composition and caries lesion histological zone) on the variation in microhardness (outcome) of resin-infiltrated artificial enamel caries lesions (RI-AEC), resulting in the formulation of the following null hypotheses:

1) Hypothesis 1 (factor 1): resin infiltrant composition does not affect the outcome;
2) Hypothesis 2 (factor 2): enamel caries histological zone does not affect the outcome;
3) Hypothesis 3 (interaction): resin infiltrant composition and enamel caries histological zone do not affect the outcome. In other words, as one goes deeper in the AEC the hydrolytic degradation of resin infiltrants is not affected.

Preparation of bovine enamel blocks
In total, 104 bovine incisors were used, which were divided into four groups (n=26). Each incisor was sectioned with diamond discs under water irrigation, obtaining blocks of bovine buccal enamel 5×5 mm. Waterproof silicon carbide paper (grade 320) were used to plan the dentin back part of the blocks, and then waterproof silicon carbide papers of increasing grades (600, 1200, 2500, and 4000) were used to obtain a smooth and flat surface of the enamel.

A single layer of acid resistant red nail varnish (Colorama, Sao Paulo, Brazil) was applied on half of the enamel area (control area) of each specimen, so that subsequent caries induction could only occur in the other half (experimental area).

In vitro artificial enamel caries induction with biofilm
For in vitro biofilm artificial caries induction, bovine enamel blocks were suspended in the 24-well plates with the aid of orthodontic CrNi 0.7 mm wire (Morelli Ortodontia, Sorocaba, Brazil). In order to fix the device to the plate, sculpting wax was used, which assisted in the fixation of the device in the plate cover. The plates were sterilized in ethylene oxide.

A smear of UA 159 strain of Streptococcus mutans was placed on a petri dish with mitis-salivarius agar (MSA) medium, which was placed in microaerophilia for 24 h. Then, bacterial colonies were collected with a loop and placed in brain heart infusion (BHI; Brain Diffusion Laboratories, Merck, Darmstadt, Germany) in a falcon tube, returning to microaerophilic for 24 h, with a culture medium without microorganisms for sterilization control and growth.

Subsequently, 20% sucrose was diluted in sterile BHI to obtain a concentration of 1% in the culture medium. In order to obtain 1 mL of the solution in each well, the following equation was used to determine the volume of sucrose to be used:

Absorbance was measured at the wavelength of 550 nanometers (A550 nm). Prior to reading, the device was reset to zero with the pure BHI value. The obtained value of BHI with inoculum grown after 24 h was the C1 of Eq. [1]. The bacterial starter concentration (C2) had a fixed value of A550=0.05. In this way, it was calculated the volume of this solution (V1) to be added to the previously prepared BHI+sucrose solution, so that the final volume (V2) needed to fill all the wells could be determined.

A volume of 1 mL of the final solution was placed in each well, placed in microaerophilia. and after 48 h the specimens were removed from the plate and washed with saline solution, resulting in artificial enamel caries lesion (ICDAS score 1).

Microradiographic analysis
All specimens were analyzed under digital microradiography in a X-ray inspecting machine (PCBA Inspector, General Electric, Wunstorf, Germany), operating with 47 V and 93 µA, to confirm the presence of the carious lesion.

Infiltrant preparation
A number of four different types of infiltrants were tested in this study. Three of them were experimental ones, and the other one was a commercial infiltrant (Icon, DMG, Wunstorf, Germany). The experimental ones were prepared with the following monomers bought from Sigma-Aldrich, St. Louis, MO, USA : triethylene glycol dimethacrylate (TEGDMA), ethoxylated bisphenol A glycidyl dimethacrylate (Bis-EMA), diurethane dimethacrilate (UDMA), 2-hydroxyethyl methacrylate (HEMA), camphorquinone (photosensitizer, 0.5 wt%), aminoethyl methacrylate (co-initiator, 1 wt%), and butylated hydroxytoluene (inhibitor, 0.1 wt%), as described before (Araújo et al., 2013). The following experimental infiltrants were used: Icon (IC); infiltrant with TEGDMA (60%), UDMA (20%), and HEMA (20%) (TUH); TEGDMA 60%+UDMA 20%+HEMA 20% (TH); TEGDMA 80%+HEMA 20% (TH); and TEGDMA 75%+Bis-EMA 25% (TB). Icon is a TEGDMA-based infiltrant, according to the manufacturer.

Resin infiltration
Prior to resin infiltration, enamel caries lesions were conditioned with 37% phosphoric acid gel (Vigodent, Rio de Janeiro, Brazil) for 60 s, following previously published protocol10). Then, they were rinsed with water jet and dried with a jet of air for 15 s. The resin infiltrant was applied only once with the aid of microbrush and left for 60 s to penetrate into the lesion. It was then photopolymerized for 60 s using light intensity of 1,000 mW/cm² (Free Light 2, 3M Espe, St. Paul, MN, USA). The following resin infiltrants were used: Icon (IC group); infiltrant with TEGDMA (60%), UDMA (20%), and HEMA (20%) (TUH group); infiltrant with TEGDMA (80%) and HEMA (20%) (TH group); and an infiltrant with TEGDMA (75%) and Bis-EMA (25%) (TB group).
perpendicularly to the surface, including both sound and infiltrated areas. The cross-sectional surface was polished using silicon carbide paper with various grades (1200, 2500, and 4000).

Transverse microhardness
Microhardness was performed at the sectioned surface, perpendicular to the original surface of the artificial enamel caries lesion. Analysis was performed using a Vickers indenter tip micro hardness tester (HMV II, Shimadzu, Kyoto, Japan), applying a load of 200 g for 10 s\(^{12}\), by a single examiner. Analysis was performed at two internal histological zones: at 30 µm from the surface (in the surface layer), and at 90 µm from the surface (in the bottom of the lesion body). A mean Vickers Hardness Value (VHN) of three indentations was obtained at each location. Measurements were performed at two time intervals: after 24 h of (time necessary for cross-sectioning, polishing, and photographing under fluorescence microscopy) the caries infiltration procedure and after a 21 days’ period of sample degradation by immersion in distilled water (pH 7.0). The outcome was the percentage difference between the final (\(\text{TMH}_f\)) and baseline (\(\text{TMH}_0\)) transverse microhardness values (\(\Delta\text{TMH}\%\)), as calculated by:

\[
\Delta\text{TMH}\% = \left( \frac{\text{TMH}_f - \text{TMH}_0}{\text{TMH}_0} \right) \times 100
\]

Considering the two factors (resin infiltrant composition and caries lesion histological zone), the groups (\(n=26\)) were distributed as described in Table 1.

Fluorescence microscopy
The sectioned surface of each resin-infiltrated artificial enamel caries lesion was analyzed under fluorescence microscopy in order to validate the penetration of the resin infiltrant into carious enamel before the first microhardness measurements. The autofluorescence property of the resin infiltrants was explored, enabling it to identify infiltrated (fluorescent carious enamel) and non-infiltrated (non-fluorescent carious enamel) parts. It was used a fluorescence microscopy technique using an epi-light system coupled to the tube of one of the eyepieces of an optical microscope (Axioskop 40, Carl Zeiss, Jena, Germany). The system (Through-eye tube epi-fluorescence illuminator, Mightex, Boston, MA, USA) was composed of 470 nm excitation filter, 510 nm emission filter, using 4× and 10× objectives, and a digital camera (Nikon D7000, Nikon, Tokyo, Japan).

Statistical analysis
This study tested a hypothesis of difference using two factors: resin infiltrant composition (4 unpaired groups) and caries lesion histological zone (two paired groups). Sample size was determined a priori, using a statistical approach that considers the largest expected difference between any two groups\(^{13}\). Based on a pilot study, we used an effect size (Hedge’s G) of 0.8, a two-tailed 5% significance level, and statistical power of 80% for unpaired groups, resulting in a sample size of 26 samples per group.

The microhardness data were analyzed using a two-way ANOVA mixed model (mixed because factors 1 includes unpaired groups and factor 2 includes paired groups) test (with a 5% significance level), which tested the three hypotheses described above.

The normality of the data and the homogeneity of the variances were tested and confirmed. Data presenting variations of ±2 in both kurtosis and skewness were considered as normally distributed\(^{14}\). Data presenting a ratio of up to 3 between the largest and the lowest variances were considered as homogeneous\(^{15}\).

In the post hoc test the unpaired \(t\)-test was used for homogeneous and non-homogeneous variances (significance level of 5% two-tailed) for comparison between pairs of groups. The paired \(t\)-test was applied to compare related groups (data from two histological zones of the same sample). No correction (e.g.: Bonferroni correction) was made for the significance level because all analyses were planned in advance to the experimental phase\(^{16}\). Statistical significance, effect size (Hedge G) and its 95% confidence interval, and statistical power were calculated for all cases, following equations described elsewhere\(^{13}\) and recent recommendations from the American Statistical Association\(^{17,18}\).

| Group 1 (IC30) | Icon\(^6\) in the superficial layer (at 30 µm from the enamel surface) |
|---------------|-------------------------------------------------------------------|
| Group 2 (TUH30) | TEGDMA/UDMA/HEMA-based infiltrant in the surface layer            |
| Group 3 (TH30)  | TEGDMA/HEMA-based infiltrant in the surface layer                 |
| Group 4 (TB30)  | TEGDMA/Bis-EMA-based infiltrant in the surface layer               |
| Group 5 (IC90) | Icon\(^6\) at the bottom of the lesion body (90 µm from the enamel surface) |
| Group 6 (TUH90) | TEGDMA/UDMA/HEMA-based infiltrant at the bottom of the lesion body |
| Group 7 (TH90)  | TEGDMA/HEMA-based infiltrant at the bottom of the lesion body      |
| Group 8 (TB90)  | TEGDMA/Bis-EMA-based infiltrant at the bottom of the lesion body   |
RESULTS
Typically, artificial enamel caries lesions presented a thin surface layer and a depth of 100 µm under microradiography (Fig. 1). The visual aspect was consistent with an ICDAS score 1. Infiltration of the infiltrates throughout the entire lesion body was confirmed in all samples by fluorescence microscopy (Fig. 1). The visual aspect was consistent with an ICDAS score 1. Infiltration of the infiltrates throughout the entire lesion body was confirmed in all samples by fluorescence microscopy (Fig. 2).

Descriptive data (mean and standard deviation) of the outcome are shown in Table 2. After water immersion for 21 days, most groups presented a decrease of ~25% in microhardness, while groups TB30 and TB90 that presented less than 10% loss of microhardness.

The two-way mixed model ANOVA results are described in Table 3. The null hypothesis 1 was rejected ($p<0.00001$), showing that the effect of infiltrant composition was significant, with an effect size of 0.183, which is moderate, and statistical power of 99.9%. The null hypotheses 2, there was no significant influence of caries zone on hidrolytic degradation of the infiltrant

| Groups | IC30 | TUH30 | TH30 | TB30 | IC90 | TUH90 | TH90 | TB90 |
|--------|------|-------|------|------|------|-------|------|------|
| Mean (SD) | -24.8 (19.1) | -24.9 (17.5) | -24.2 (16.8) | -4.3 (23.7) | -27.3 (17.5) | -26.9 (13.7) | -27.8 (14.4) | -9.0 (18.7) |
Table 3  Two-way mixed model ANOVA test results for the effects of infiltrant composition and caries histological layer on ∆TMH%

| Factor                        | Sum of squares | Degrees of freedom | Mean squares | F       | p value | η²   | Power |
|-------------------------------|----------------|--------------------|--------------|---------|---------|------|-------|
| Infiltration composition      | 14,531.349     | 3                  | 4,843.783    | 15.063  | 7.07×10⁻⁹ | 0.183 | 0.999 |
| Caries histological layer     | 525.365        | 1                  | 525.365      | 1.634   | 0.203   | 0.209 |       |
| Interaction                   | 56.325         | 3                  | 18.775       | 0.009   | 0.998   | 0.007 | 0.209 |

The results of the post-hoc analysis between pairs of groups are shown in Table 4. The groups TB (TB30 and TB90) presented lower decrease in ∆TMH% than the other groups, with large effect sizes (Hedges G from 0.83 to 1.19) and high power (from 84 to 99%). For the comparisons that do not involve the TB groups, the effect size was very small (from 0.01 to 0.22).

DISCUSSION

The present study provides evidences indicating that hydrolytic degradation of resin infiltrant is affected by infiltrant composition, but not by the histological layer of the artificial enamel caries lesion. The results have implication in both the esthetic and caries preventive outcomes of caries infiltration.

The protocol used for the application of the resin infiltrants was different from the one recommended by the manufacturer of the Icon infiltrant². The pro-
infiltration procedures were standardized for all infiltrants, as performed previously, in order to minimize the effect of biases in the comparisons. The etchant used was 37% phosphoric acid gel because our AEC lesion presented thin surface layer (Fig. 1) and 37% phosphoric acid is less aggressive for removing surface layer than hydrochloric acid, which is used clinically. Only a single application of each resin infiltrant was performed because it has been shown (in a pilot study) that this procedure resulted in complete penetration of the artificial enamel caries lesion created by our microbiological method.

The pores of resin-infiltrated enamel caries lesions are filled by a mixture of organic matter, firmly bound water, air, and resin infiltrant. The resin infiltrant located in the outermost superficial layer of enamel caries lesion is prone to water degradation by water from two sources: firmly bound water in the enamel pores and water from saliva. By immersing transverse sections of enamel caries lesions in water, all histological layers were exposed to both enamel pores’ firmly bound water and water from the external environment. Thus, the two enamel caries histological layers were exposed to relatively similar amounts of water during the experiment.

Hardness measurements were performed at different depths of lesion because it has been reported that resin penetration deep in the body of the lesion is more deficient than in other histological zones. The volume and size of pores varies among enamel caries histological zones, and it is known that such variation influences enamel permeability and interfere in the amount of resin infiltrant penetrated into hypomineralized enamel. In addition, presence of trapped air at the bottom of the body of the lesion may reduce resin penetration and may facilitate the water percolation and also, the hydrolytic degradation of the polymers. The fact that our enamel caries lesions were uniform and shallow (Fig. 1), and completely filled by resin infiltrant (Fig. 2) might explain why the null hypothesis related to the enamel caries histological layers and the interaction hypothesis were not rejected (Table 3).

The lowest reduction in microhardness upon water immersion was observed with TB infiltrant (TB90 and TB90 groups). All resin infiltrants tested here have been previously compared regarding various physical properties, and the results that are relevant for this study are the following: (i) the experimental infiltrants (TUH, TH, and TB) presented lower degree of conversion and penetration depth into AEC than Icon; (ii) the elastic modulus of TUH was higher (1.01 GPa) than Icon (0.90 GPa), TH (0.70 GPa), and TB (0.95 GPa); and (iii) Icon presented much lower hardness (6.54 KHN) than TH (8.86 KHN), TB (13.08 KHN), and TUH (14.74 KHN). It is known that the composition of the infiltrants directly influences their properties. TEGDMA is a highly hydrophilic, low viscosity monomer with a high degree of conversion. HEMA is also hydrophilic, and contributes to decrease hardness of TEGDMA-based infiltrant when compared with blends that contain hydrophobic monomers. Bis-EMA is a hydrophobic monomer, and has a lower water degradation than the other monomers used in this study. UDMA is also a hydrophobic monomer, but, compared to Bis-EMA, it has a smaller molecular size, higher viscosity, and is more linear. Such monomer properties could explain the ∆TMH% obtained with the TB infiltrant.

The degradation of resin infiltrant in resin-infiltrated enamel caries contributes to the increase of the pore volume not filled by infiltrant (the sum of the organic, firmly bound water, and air volumes). Most likely, resin degradation creates a pore volume that will be filled by water. With its lower refractive index and higher permeability, water incorporated into resin-infiltrated enamel caries lesion might contribute to increase both enamel opacity (rendering the enamel caries lesion more clinically visible; i.e. less esthetically appealing) and permeability (contributing to caries lesion progression). It has been recently shown that artificial enamel caries lesion infiltrated with Icon was not protected from caries progression in vitro. Enamel pores filled with water provide the most likely pathways for acid diffusion in resin-infiltrated enamel caries. Water degradation of resin infiltrant might contribute to the increase enamel pores filled with water.

While hydrophilic monomers favor deep penetration of carious enamel, favoring penetration depth, their water degradation might compromise the outcomes of caries infiltration. Hydrophobic monomer, on the other hand, are more likely to have air in the nearest enamel pore environment. Among the component volumes of resin-infiltrated enamel caries lesions, both the resin-infiltrated and the air-filled volumes are not included in enamel permeability, thus contributing to the protection against caries progression. In this context, the slower, shallower penetration of resin infiltrants rich in hydrophobic monomers compared to infiltrants rich in hydrophilic monomers might not be deleterious to the protection against caries progression. By being surrounded by air volume, hydrophobic monomers might prevent the contact of water with the infiltrant, thus reducing water degradation. Studies of the ultrastructure of the infiltrated pore are needed to unravel this issue. Future studies should investigate whether infiltrating with hydrophobic monomers results in increased air volume in the enamel carious lesion, whether this air volume is stable after prolonged exposure to water, and what is the rate of enamel permeability reduction compared to infiltrating with hydrophilic monomers and Icon.

**CONCLUSION**

We conclude that infiltrant composition affect significantly its hydrolytic degradation, concerning ∆TMH%, but it is not affected by the enamel caries histological zone. The lowest decrease in ∆TMH% was observed for the TB infiltrant.
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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or nonfinancial.

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