Evaluation of an experimental remineralizing agent for repairing enamel surfaces

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ABSTRACT
The best material for repairing enamel surface defects is one very similar to the original enamel and which interacts with natural remineralization mechanisms. It does not repair extensive damage, so in order to fill large defects,external help is required using phosphocalcic ceramic composites that activate salivary remineralization efficiently though on smaller in scale. Effective adhesion of the repair may depend on the amount of aqueous fluids present in the enamel, which apparently enable nucleation and growth of new minerals to ensure adhesion and stability. The amount of fluids is governed by osmotic pressure. This study evaluated the influence of two osmotic pressure values of isotonic and hypotonic saliva and two modified remineralizing agent compositions: combinations of "conditioner" and "remineralizing agent" in proportions of 90%: 10% (A) and 50%: 50%(B), on filling artificial cracks. Results were evaluated by profilometer, stereomicroscope and confocal laser microscope. A 22 factorial design and a logistic model for statistical analysis were used. Only the composition of the mineralizing agent had a significant effect on efficiency in repairing defects. Compositions A and B both repaired dental enamel defects, but composition B presented higher levels of repair and more compact deposits as observed under stereomicroscope.

Key words: *Dental enamel, biomaterials, calcium phosphates, tooth remineralization.*

Evaluación de un agente remineralizante experimental reparador de superficie de esmalte

RESUMEN
El mejor material para reparar defectos superficiales del esmalte es uno muy similar al original y que este interactúe con los mecanismos naturales de remineralización. Este no arregla daños extensos por lo que se requiere de una ayuda externa para rellenar defectos grandes con un material que active la remineralización salivar que sea eficiente pero de menor alcance. Para esto se emplearon cerámicas compuestas principalmente fosfocálcicas. La adhesión efectiva de la reparación puede depender de la cantidad de fluidos acuosos existentes en la porosidad del esmalte pues aparentemente permiten la nucleación y crecimiento de nuevos minerales para asegurar adhesión y estabilidad. La cantidad de fluidos está gobernada por la presión osmótica. En este estudio se evaluó la influencia que tienen dos valores de presión osmótica de la saliva isotónica y hipotónica y dos composiciones de agente remineralizante modificado: condicionador y agente remineralizante en composiciones de 90%/10% (A) y 50%/50%(B) respectivamente, sobre el llenado de grietas artificiales por perfilometría, estereomicroscopio y microscopía confocal láser. Se trabajó con un diseño factorial 22 y tratamiento estadístico: modelo logístico. Solamente la composición de la sustancia remineralizante tuvo efecto significativo en la eficiencia para reparar defectos. La composición tiene un efecto reparador sobre los defectos del esmalte dental en sus dos composiciones, no obstante, la composición 50%/50% presenta niveles más altos de reparación y forma depósitos que al estereomicroscopio se observan más compactos.

Palabras Clave: *Esmalte dental; Materiales biocompatibles; Fosfatos de calcio; Remineralización dental.*

INTRODUCTION

Dental enamel is a bioceramic composite which consists of 96% minerals and 4% organic material (proteins) and water [1,2]. Because it lacks cells, rather than being considered a tissue, it is considered to be a highly mineralized extracellular substance incapable of regenerating itself when it suffers attacks [1,3].

Enamel may suffer superficial defects such as infractions in response to mechanical over-exertion or extreme conditions to which it is subjected in the oral cavity. To treat such injuries, reparative techniques have been used,[4]including restorative materials with different retention mechanisms[5] such as amalgam, metal alloys, ceramics, and composite resins combined with dental adhesives[6,7], all of which have provided acceptable solutions for preserving tooth integrity. However, small, shallow lesions that are thicker than 50μm[8] do not warrant the use of preparations that destroy even more dental structure and lead to the use of restorations that form interfaces with the dental substrate and facilitate microfiltration and tooth decay. Knowledge of the composition of enamel and biomaterials has driven the search for more conservative solutions to the problem of loss in tooth integrity.

Different bioceramics have been proposed for clinical use in view of their biocompatibility, bioactivity and dissolution rates. Supersaturated solutions of calcium, phosphate and magnesium ions[9,10], fluorapatite-gelatin[11], electrodeposition[12], apatite[13] and fluorapatite cement have been studied and proposed for tooth enamel repair[14]. Many studies of these materials are currently still in the *in vitro* phase, so their effects in clinical conditions are as yet unknown.

Agents containing mainly calcium phosphates, such as a "modified conditioner" and a "remineralizing agent", which have been tested *in vitro* on enamel crack defects, decreased the size and number of these defects without creating an interface. The "modified conditioner" produced many adherent deposits of irregular appearance and different color of the enamel[15,16], while the "remineralizing agent" created less abundant deposits and had a clear appearance and regular pattern[17,18]. Experiments were conducted with each agent at two different time points on moist, freshly extracted teeth. The agents were then tested on dry teeth one year after extraction. Better results were obtained with wet teeth than with dry teeth. As a general result of the experiments, it was suggested that the difference in the results was due to the moisture level of the specimens [15-18].

The seemingly complementary properties of the two ceramics - "remineralizing agent" and "modified conditioner",in addition to their ease of handling and the low cost of their components, led to the proposal of combining them as a third substance, Modified Remineralizing Agent (MRA), which combines properties of the original components regarding volume, regularity and color of the deposits formed. Different proportions of the two ceramics in the combination yield different results, and must therefore be defined.Two compositions were selected and called MRA_1 (90% remineralizing agent and 10%modified conditioner) and MRA_2 (50% and 50%). The effects of these two MRA compositions and two moisture contents on the efficiency of the repair process on groove-shaped enamel defects (more or less deep straight cuts) in tooth enamel were studied. The aim of these experiments was to determine whether the level of enamel repair depended on saliva composition (osmotic pressure), modified remineralizing agent (MRA) composition, or the interaction between them. This study seeks to advance the understanding of the behavior of the material under controlled laboratory conditions.

MATERIALS AND METHODS

Sample collection

With prior approval from the Ethics Committee of the School of Dentistry at Universidad Nacional de Colombia (CIE-00017-11) and signed informed consent from the donor patients, aged 15 to 45 years, 104 human teeth without hypoplasia, fractures, endodontic treatment, rehabilitation, bleaching or vestibular restorations were collected.

Sample pre-treatment

Following the Tooth Bank standards of the School of Dentistry at Universidad Nacional de Colombia, the teeth were transported, cleaned, disinfected and preservaded in 0.5% chloramine-T at 4°C[19]. The middle third of the vestibular surface was established as the study zone, and delimited by four marks in a square made with a round ¼ carbide bur, 17839-SSWHITE, with a high-speed NSK (at 200,000 rpm) handpiece. A cutter was used to create groove-shaped enamel defects in mesiodistal direction, 200 μm to 400 μm deep and 100 μm to 260 μm wide. The profiles and dimensions of the defects were established using a Veeco profilometer, Dektak model; a Nikon SMZ 800 C-DS stereomicroscope and an LSM 700/Zeiss confocal laser microscope (Fig.1).

Following the experimental design in a symmetrical 2^2 factorial arrangement, there were two controlled factors with two levels each: Compositions of the modified remineralizing agent $MRA_1$and MRA_2, and isotonic (IS) and hypotonic (HS)saliva. The two different MRA compositions contained different percentages of preexisting components (products

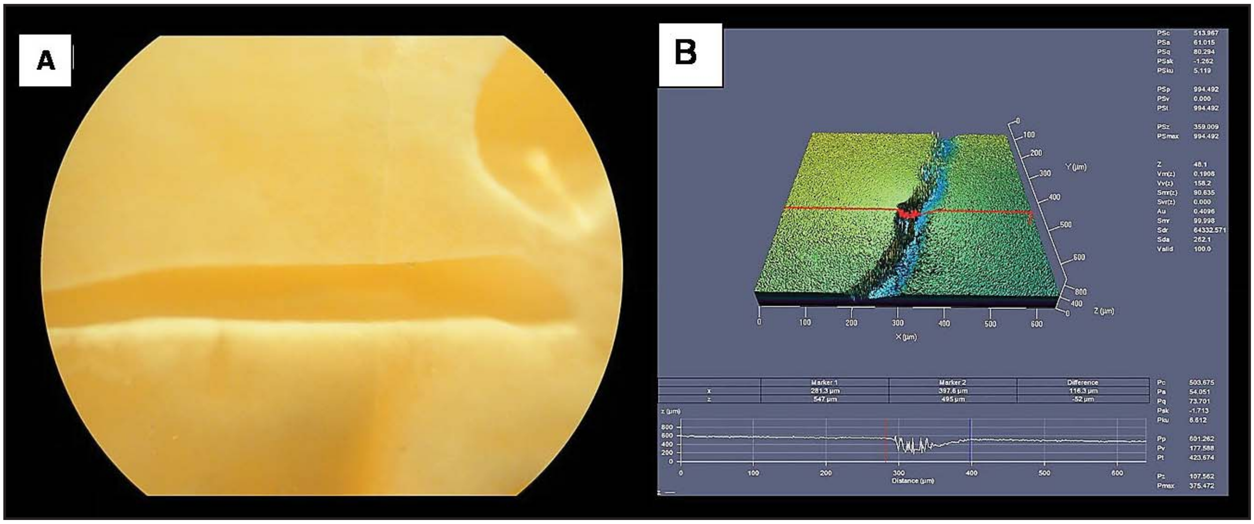

Fig. 1: A and B Groove-shaped enamel defects200-400 µm deep and 100- 260 µm wide were created in the mesiodistal direction. Defect profiles and dimensions were established using stereomicroscope and confocal laser microscope.

of the ceramics laboratory, Department of Chemistry, Universidad Nacional de Colombia) and saliva composition was established by varying its osmotic pressure. Specimens were randomly divided into four paired groups of 26 samples, labeled as MRA_1-IS, MRA_1-HS, MRA_2-IS and MRA_2-HS, where IS and HS are isotonic and hypotonic saliva respectively, according to the corresponding combinations of treatment effects.

Sample treatment

To evaluate the effect of the osmotic pressure of the moist environment on the outcome of filling defects, teeth were submerged in artificial isotonic (IS) or hypotonic (HS) saliva for two weeks, then washed with a toothbrush under running water, after which they were subjected to surface abrasion with number 1000 silicon carbide sandpaper, washed in water with a soft tooth brush and cleaned with 2-propanol to remove impurities. To evaluate the efficiency of the MRA compositions, MRA_1 and MRA_2 were applied to randomly selected teeth for six hours, with the treatment area isolated from the environment. After the treatment time, the teeth were washed again with a brush and running water to remove any non-adherent deposited material. Teeth were observed and photographed via stereo-microscopy at 4X magnification. A previously calibrated observer, with Kappa 1 intra-examiner reproducibility, assessed treatment results under a stereomicroscope by classifying the filling of defects into three levels: **Low**, when part of the floor of the created defect was visible (not covered with filling material); **Medium**, when the filling did not reach one or all of the external boundaries of the defect, but the floor was not visible; and **High**, when the filling level of the defect reached or exceeded the external boundaries.

Statistical analysis

Because the response variable was binary, a logistic model was used to identify the effect of treatments. Different analyses were performed using generalized linear models to determine the effects of saliva composition (osmotic pressure) and composition (MRA_1 or MRA_2) on the level of tooth repair. The chosen statistical hypotheses of interest (MRA composition, saliva, and their interaction) were considered significant when $p< 0.05$. Goodness of fit was verified with statistical procedures based on properties of the chi-squared distribution, and the quality of fit of the proposed model was validated through a graphic review of Pearson and Deviance residuals. The information obtained was processed using R software, version 11.1.

RESULTS

All treated teeth presented some degree of repair, and only one tooth presented a low level of repair. This tooth was eliminated, and only medium and high levels of repair were considered; thus, the analysis focused on modeling the probability that the tooth would have a high level of repair. A greater number of teeth was found to have a high level of repair (76% of the total). The proportion of teeth with medium and high level of repair was the same for each treatment; however, teeth treated with composition MRA_2 (50%-50%) seemed to attain a higher degree of repair (Table 1).

Fitting the logistic model to the interaction showed no statistical significance ($p = 0.43$), meaning that when salivary osmotic pressure varied, whether or not the MRA composition was constant, there was no change in the level of repair.Thus, the final definitive study model additively incorporated the effects of saliva composition and remineralizing agent only.

For the model with interaction, changing the osmotic pressure did not statistically influence the level of repair, given that the $p=0.82$ value is very high. In contrast, significant differences were detected for MRA ($p< 0.05$), as shown in Table 2.

With regard to differences in the effects achieved with the two MRA compositions, MRA_2 was found to be more likely to achieve a high level of repair. Using the logistic model, the probability of repair for each treatment is listed in Table 3. Best outcomes were achieved with hypotonic saliva, with 0.85 probability.

The p-value associated with Pearson's chi-squared statistic was 0.28, suggesting a good fit of the model and validating the conclusions reached above. Diagnosis of the model residuals shows no unsuitability problems for the adjusted model.

All teeth showed some degree of defect coverage. Fig.2 shows characteristics of enamel defect fillings under a stereomicroscope, a confocal laser microscope and scanning electron microscopy (SEM). Composition MRA_2 provided more regular and dense filling (Fig. 3A) than MRA_1 (Fig. 3B). Observation of the agent deposited on the enamel defects revealed spherical particles (Fig. 3C) and irregular elongated shapes (Fig. 3D).

Table 1: Number of teeth according to repair level and treatment. Specimens with medium or high levels of repair.

	Number of teeth according to repair level	
Treatment	**Medium**	**High**
MRA1-IS	9	17
MRA1-HS	7	18
MRA2-IS	3	23
MRA2-HS	5	21
Total	24	79

MRA1-IS: Modified Remineralizing Agent (1,2) – isotonic saliva
MRA2-HS: Modified Remineralizing Agent (1,2) – hypotonic saliva

DISCUSSION

Most studies in the literature report promising results regarding the remineralizing capacity of the agents proposed for early management of artificial cavities[9,20-25]. Unlike the previous studies, but following similar principles, this study sought to test the repair capacity of MRA as compositions MRA_1 and MRA_2 on mechanically created defects, toward the ultimate goal of creating an efficient material that can be used in clinically visible lesions.

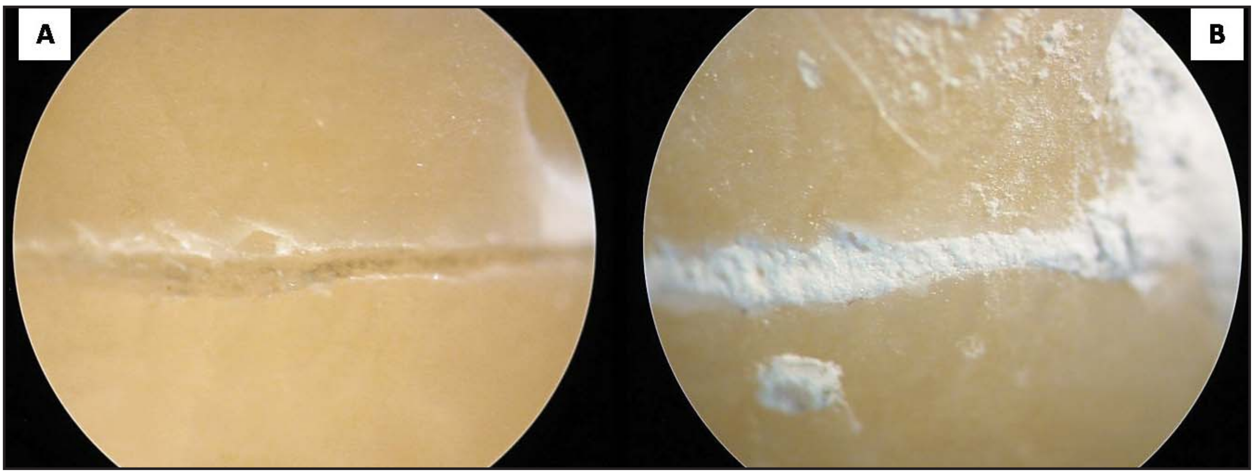

Fig. 2: A and B show characteristics of enamel defect before and after filling with MRS under a stereomicroscope.

Table 2: Statistical analysis of the model.

Variable	Statistic	Degrees of freedom	p-value
Saliva composition (osmotic pressure)	0.0549	1	0.82
Remineralizing agent composition	43.437	1	0.04*
OP Interaction and Composition		1	0.428

OP: osmotic pressure

Table 3: Logistic Model. Probability of repair for each treatment.

Probability of repair	MRA1-IS	MRA1-HS	MRA2-IS	MRA2-HS
High repair probability	0.685	0.661	0.839	0.853
Medium repair probability	0.315	0.339	0.161	0.147

MRA1-IS: Modified Remineralizing Agent (1,2) – isotonic saliva
MRA2-HS: Modified Remineralizing Agent (1,2) – hypotonicsaliva

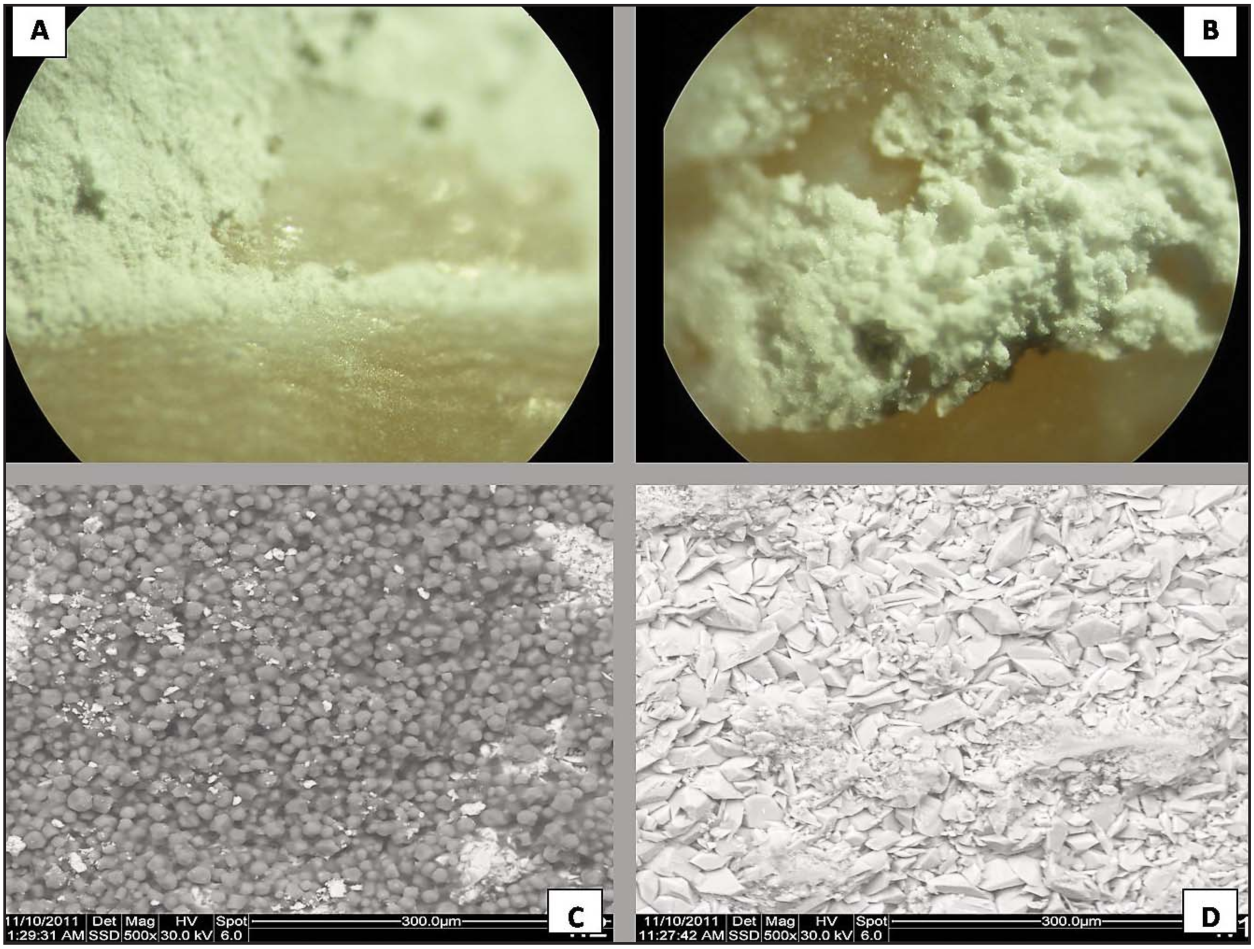

Fig. 3: A, MRA2: more regular and dense filling was observed. B, MRA1:irregular and no dense filling. Agent deposited on the enamel defects: C, spherical particles and D, irregular, elongated shapes.

The agent tested in this study is composed of constitutive ions from enamel, mainly phosphates and calcium, enabling it to bind chemically to the enamel substrate, based on the principle of secondary nucleation. Approximate particle size is 5-15µm, determined by SEM at 500x, and particles have irregular, elongated, spherical shapes that may correspond to amorphous phases or different degrees of crystallinity. Because ceramics are more durable than polymers, which hydrolyze and degrade over time, it is advantageous to use purely ceramic compositions. Additionally, because the ceramics are mainly phosphocalcic, they play an active role in remineralization with natural saliva and may simultaneously lead to remodeling of the structure and surface porosity according to the Ostwald ripening principle.

Based on preliminary tests, an application time of six hours was established, which was the shortest time that allowed abundant formation of adhesive deposits within and around the defects.In 2001, Eisenburger et al. produced erosions with citric acid using the same time period andobserved decreased lesion depth after storage in artificial saliva and exposure to ultrasonic cleaning, suggesting that strong bonds were achieved between the dental substrate and the applied material[26]. This is desirablefor agents used for repairing the surface of hard dental tissue. Li et al. 2008 observed a weak bond when using blocks of hydroxyapatite nanoparticles, perhaps due to differences in size and development time of the material formed compared to those from original hydroxyapatite enamel[3]. Our study tested whether the agent was easily removed by mechanic brushing with moderate pressure under running tap water for five seconds, after which the permanence of the material deposited on the tooth surface was observed, particularly within the defects. This permanence could be explained based on nucleation principles, with the defect area having high binding energy compared to the surfaces.

Many studies have used different methods to demonstrate the repair capacity of biomimetic agents[21, 24, 27, 28] such as supersaturated ionic solutions that favor the precipitation of calcium and phosphate ions[29]. These solutions appear to be more efficient than saliva itself, possibly due to the organic composition of saliva having an inhibitory effect on crystal nucleation and growth[30]. Immersing teeth with artificial cavities in synthetic hydroxyapatite solution with a calcium/phosphate ratio of 1.63 showed repair capacity not only in the enamel surface but also in the depth of the lesion, unlike the same treatment with human saliva (with or without fluoride), in which the effect was limited to the surface[31]. Studies using toothpaste with hydroxyapatite-carbonated nanocrystals observed formation of a coating on the tooth enamel, with inferior crystallinity to that of the enamel but adequate capacity to repair the surface defects described[20]. Using natural caries lesions, Wei et al.[14] tested a fluorapatite cement as a repair material for tooth enamel cavities previously acid-etched with 17% phosphoric acid for 30 minutes. They achieved a strong bond to the enamel with no apparent gap and the structural composition of apatite.

Other studies seeking the same repair effects achieved with mentioned agents and using ionic agents applied on superficial cracked tooth enamel, observed the formation of adhesive deposits that completely or partially covered these defects, with effectiveness that appeared to depend on the moisture used[15-18]. Our study mixed remineralizing and conditioning agents in two different proportions to prepare compositions MRA_1 and MRA_2,which were placed in direct contact with groove-shaped surface defects of teeth. Both these compositions provided mostly high filling levels. Although no statistically significant difference was observed between treatments in relation to reparative capacity, teeth treated with composition MRA_2 (50% and 50%) had the highest level of defect repair. This could be because the agent is composed of two precursor agents in equal proportions, which may increase the potential of both: the filling volume of the restorative material and the fineness, low volume and substrate-binding characteristics of the conditioning agent, which had been pretested as a bracket-bonding material. Such combined qualities couldalso explain why this composition has an aesthetically acceptable appearance, perhaps because the fine particles cover spaces that are left bycompositions of larger volume.

The fact that there does not appear to be a significant effect of the aqueous medium to which specimens were previously subjected on the restorative capacity of MRA_1 and MRA_2 compositions in this study may indicate thatthe saliva compositions used here might not be different enough to establish their influence.

The agent deposited on the tooth surface is chalk-white in color and has a porous appearance compared to tooth enamel. The white color is due to the fact that the agents applied are colorless crystalline materials which produce the full range of visible colors due to the multiple angles at which they diffract light, perceived together by the eye as white. Its difference in color of human enamel is due to the orientation, size and shape of the crystals and to the fact that spaces between enamel prisms are filled with organic material and the glass phase of calcium phosphate crystals, which increase translucency, while in the repair area there are unfilled pores. Differences may also be related to the short duration of the mineral formation process compared to the biomineralization of hard tissues, which does not allow sufficient ripening to achieve a higher degree of crystallinity. Further studies should consider filling pores in the restored area with biomimetic glassy material which in addition to decreasing porosity, may improve appearance and color.

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