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Effect of Protein-Based Treatment on Chemical Composition, Hardness and Bond Strength of Remineralized Enamel

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This study evaluated the chemical composition and microhardness of human enamel treated with an Enamel Matrix Derivative (EMD) solution, and the bond strength between composite resin and this enamel. Thirty human enamel samples were randomly divided into three groups: Untouched Enamel (UE), Demineralized Enamel (DE) and Demineralized Enamel Treated with EMD (ET). DE and ET groups were subjected to acid challenge and ET treated with EMD (EMD was directly applied over conditioned enamel and left for 15 min). Samples from each group (n=4) had chemical composition assessed through to attenuated total reflectance Fourier transform infrared (ATR-FTIR). Knoop microhardness of enamel samples from each group (n=10) was measured. For the microshear bond strength, the samples were etched for 30 s, and the adhesive was applied and cured for 10 s. Two matrixes were placed on the samples, filled with Filtek Z350 XT composite and cured for 20 s, each. The matrix was removed, and the microshear bond strength of each group (n=10) was tested. Data were subjected to Kruskal-Wallis test (for microhardness), to analysis of variance and to Tukey's test (for microshear bond strength); (α=0.05). FTIR results have shown phosphate (hydroxyapatite indicator) in 900-1200 cm^{-1} bands in the UE and ET groups, which were different from the DE group. Microhardness and microshear analyses recorded higher statistical values for the UE and ET groups than for DE. EMD application to demineralized enamel seems to have remineralized the enamel; thus, the microhardness and bond strength was similar between UE and ET groups.

Key Words: enamel matrix derivative, chemical composition enamel, microhardness, microshear bond strength.

Introduction

Human tooth enamel is mostly composed of calcium- and phosphorus-rich minerals. Apatite crystals' development during amelogenesis is directed by an organic matrix made of proteins, mainly of amelogenins. Among other functions, amelogenins are responsible for regulating the mineralization process and for organizing apatite crystals into juxtaposed prisms (1). Certain external factors may lead to partial loss of enamel, whose restoration process should meet some requirements in order to assure its durability and aesthetics. Enamel regeneration has been investigated as an alternative way to replace these losses. The biomimetic approach recommends adopting techniques inspired in natural processes, according to which the matrix reestablishment process guides tissue growth (2).

Methods focused on regenerating dental enamel have been tested. The most common method available in the literature regards the use of self-assembling peptides or lyophilized enamel matrix proteins (3). Enamel matrix proteins play a fundamental role in regulating enamel mineralization, but the regeneration of this tissue depends on the previous exposure of a functional amelogenin layer of its matrix (4). Such exposure can be done by acid etching the surface to be regenerated (5). The amelogenin protein molecule is typically divided into three amino acid domains, namely: central domain, C-terminus (COOH) and N-terminus (NH_2) (6). The central domain has hydrophobic proprieties, whereas both C- and N-terminus are hydrophilic (7): C-terminus acts in protein-mineral ratio and N-terminus in protein-protein ratio (1,8), although it is known that both terminus types play key roles in proteolytic processes (1).

Matrix assembly is based on the binding of free proteins to proteins on the exposed surface of the enamel. This process leads to the formation of a protein network, which is the framework for mineral deposition (8). Amelogenin molecules group themselves into oligomers, which, in their turn, organize themselves into nanospheres (7); their arrangement in a ribbon pattern forms the framework that determines the parallelism of enamel prisms (7). Such framework enables the free minerals-nucleation process in the organic matrix to enable hydroxyapatite crystals to grow (9). Although these processes can be artificially reproduced, they are physiological during amelogenesis. Some researchers point towards their possible clinical applications to rule out white spot (5,10) and erosion lesions (11,12), as well as to prevent such conditions from happening by improving enamel resistance to future acidic challenges (12,13).

Emdogain (Straumann AG, Basel, Switzeland) is a commercial enamel matrix derivative solution (EMD; mostly lyophilized porcine amelogenins), which was developed to improve the quality of bone grafts used in Implantodontics and in Periodontics. However, some researchers have successfully tested this product as enamel re-hardening (5) or biomineralization agent, since it has the advantage of being a standardized solution. Despite some studies focused on accessing the mechanical proprieties of regenerated dental enamel, little is known about its behavior when it is subjected to restorative materials. Once known that adhesion may be harmed when performed over demineralized enamel, regeneration therapy is justified as a method of improving bonding results. The aims of the current study were to evaluate EMD application to demineralized enamel, as well as to examine the chemical composition and microhardness of the enamel and the bond strength between composite resin and tooth enamel. The herein tested hypotheses were EMD use in enamel remineralization affects [1] chemical composition, [2] the microhardness of the enamel or [3] the bond strength between composite resin and tooth enamel.

Material and Methods

The study was approved by the Research Ethics Committee (CEPAE-80223617.8.0000.0108; Protocol n. 2.503.136). In total, 51 non-restored human lower third molars and caries-free human third molars were extracted. These molars did not have history of trauma, bruxism or cracks. All teeth used in the current research were extracted for orthodontic purposes and collected upon patients' consent.

Sample Preparation

Extracted molars were kept cold for no more than three months after extraction. The extracted molars were washed with water and stored in 5% chloramine-T solution at 37 °C for 5 days, until they were used. The teeth were selected, scale-cleaned and stored in freezer. One enamel sample (3x6 mm) was cut from the buccal surface of a tooth of each specimen. Samples were flatted using SiC sandpapers (#600, #1200, #2000; Norton Abrasivos, Recife, PE, Brazil) and felt disc with diamond paste (Arotec, Cotia, SP, Brazil) to obtain polished surfaces. After the polishing step was over, all specimens were placed in ultrasonic washer (Ultra Cleaner 1400; Unique, Indaiatuba, SP, Brazil) for 10 min in order to remove debris.

Knoop Microhardness was measured in a hardness testing machine (HMV-G; Shimadzu, Kyoto, Japan). Three readings were taken under load of 25 g, for 5 s, in each sample. The mean hardness of all enamel samples was calculated; samples showing values lower than, or equal to, 10% were excluded from the study to assure similar initial hardness between groups. Thus, 21 samples were discarded, whereas the remaining 30 samples were randomly divided into three groups: Untouched Enamel (UE), Demineralized Enamel (DE) and Demineralized Enamel Treated with EMD (ET).

DE and ET groups were subjected to acid challenge in buffer solution (pH5) at 37 °C, for 4 days (the solution was changed on a daily basis), whereas UE samples were stored at 4 °C, under 100% humidity condition. The ET group was cleaned in ultrasonic washer with distilled water for 10 min and etched with 37% phosphoric acid for 20 s, rinsed in water for 40 s and gently dried with air. Then, Emdogain was directly applied over conditioned enamel with its provided syringe, completely covering all the sample surface and left for 15 min. After that time, samples were again washed with distilled water for 20 s. Subsequently, all samples were stored in artificial buffer saliva solution (pH7) at 37 °C, for 7 days. The artificial saliva used was prepared by mixing magnesium chloride ($MgCl_2$, 0.2 mM), calcium chloride ($CaCl_2$, 1 mM), potassium dihydrogen phosphate (KH_2PO_4, 4 mM), potassium chloride (KCl, 16 mM) and ammonium chloride (NH_4Cl, 4.5 mM) in 20 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid) buffer. Adjust pH to 7.0 with 1 M NaOH and store the artificial saliva solution at 4 °C. Each sample was separately stored and the storage media was changed every day.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Four random samples from each group (n=4) were analyzed; the chemical composition of the enamel was investigated based on Fourier Transform Infrared Spectroscopy (PerkinElmer, Beaconsfield, UK); the spectrometer was equipped with an accessory that enabled spectrum acquisitions in Attenuated Reflectance (ATR) mode. Spectra were recorded at range of 1400-400 cm^{-1} - this procedure aimed at phosphate bands between 1200-900 cm^{-1} at 4 cm^{-1} resolution. The testing surface was positioned against the diamond crystal of the FTIR unit and pressed with a force gauge at constant pressure to enable contact. The sample was scanned 64 times in each FTIR measurement; the acquired spectrum corresponded to the mean of all scans.

Microhardness Test

The superficial microhardness in all of the enamel samples was measured once again in a hardness testing machine (HMV-G; Shimadzu, Kyoto, Japan) equipped with a Knoop-type indenter at a static charge of 25 g applied every 5 s. Three indentations (100 µm from one another) were performed in the center of each sample. The first indentation in the initial mark was made in the upper left corner; 1,500 µm on the horizontal plane and 1,500 µm on the vertical plane, and the mean values were obtained by the indentations represented the sample. The Knoop hardness

was expressed as the mean of three indentations made in the same sample.

Microshear Bond Strength Test

After the microhardness test was over, samples were subjected to microshear bond strength test. In order to do so, each sample had its surface etched with 37% phosphoric acid (dental conditioner gel; Dentsply, Petrópolis, RJ, Brazil) for 30 s, washed with air/water spray for 60 s, and dried with absorbent paper filter. After, the adhesive (Adper Scotchbond Multipurpose Adhesive; 3M ESPE, St. Paul, MN, USA) was applied to the etched enamel surface by following the manufacturer's instructions, which comprised the application of two adhesive layers, light air jet and, finally, dental curing light (Radii-cal; SDI, Baywater, VIC, Australia) for 10 s (1400 mW/cm^2).

Two 1-mm-long transparent plastic tubes (Tygon tubing, TYG-03; Saint-Gobain Performance Plastic, Maime Lakes, FL, USA) presenting internal diameter of 0.75 mm (14) were placed over the adhesive enamel surface. The tubes were filled with a bulk-fill composite resin (Filtek Z350 XT; 3M ESPE) in shade A2B, and light-cured for 20 s. Two cylinders were made over each sample and the mean bond strength of the two cylinders was calculated for each specimen. Samples were stored at 37 °C for 24 h. Subsequently, the tubes were carefully removed with a n.15 scalpel blade (Solidor, Osasco, SP, Brazil) to avoid strain induction in the composite resin. This procedure revealed a composite cylinder and exposed two cement cylinders presenting 0.38 mm^2 bond area, each.

Samples were then secured onto a microshear device adapted to a universal testing machine (EMIC DL 2000, Equipamentos e Sistemas de Ensaio, São José dos Pinhais, PR, Brazil). Steel wire (0.2 mm diameter) was used to apply load to the basis of the cylinder at 0.5 mm/min (speed) until it presented microshear bond strength fracture. Values were converted into MPa by dividing the force (kgF) by the adhesive area (cm^2). Each group comprised 10 samples (the power of test was 99%) and each sample held two cylinders, thus totaling 20 cylinders per group.

Fractured cylinders were qualitatively observed under optical microscopy (Olympus Corp, Tokyo, Japan) at 40× magnification. Fractures were classified as cohesive (composite), adhesive (interface) or mixed (presence of composite and/or enamel in the same fragment).

Statistical Analysis

Statistical analyses were performed in the Minitab 16 software for Windows 8 (Minitab, State College, PA, USA). Measurement distributions were analyzed through Kolmogorov-Smirnov normality test, which was followed by nonparametric Kruskal-Wallis test, at 5% significance level (α=0.05) for microhardness test; and by parametric analysis of variance (ANOVA) and Tukey's test, at 5% significance level (α= 0.05) for microshear bond strength test.

Results

As FT-IR spectra were acquired in ATR mode, lower transmittance values indicate higher intensity on target bands. Figure 1 shows bands of a sample from each Group (UE, DE and ET). UE and ET presented similar bands, whereas the FT-IR of DE group presented lower intensity spectra than the other groups in the 1200-900cm^{-1} band (shaded area), showing lower rates of phosphate group bonds.

Microhardness data are shown in Figure 2. There were statistically significant differences among different groups

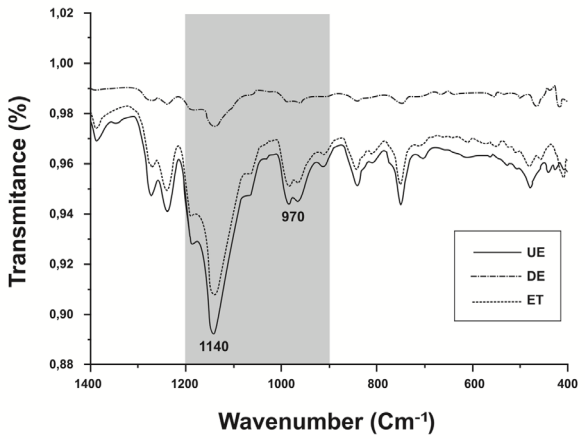

Figure 1. Bands of a sample from each Group (UE, DE and ET). UE: Untouched Enamel; DE: Demineralized Enamel; ET: Demineralized Enamel Treated with EMD.

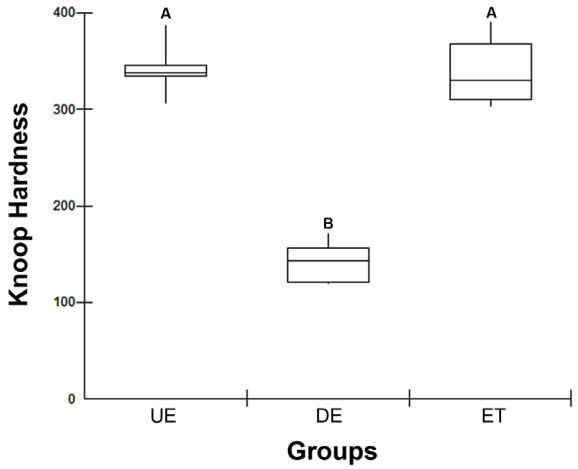

Figure 2. Microhardness data are shown in Figure 2. Different letters indicate statistically significant differences among median values, according to the Kruskal-Wallis test, at 5% significance level. UE: Untouched Enamel; DE: Demineralized Enamel; ET: Demineralized Enamel Treated with EMD.

(p=0.0001). The microhardness medians recorded for UE and ET were significantly higher than that of DE.

Microshear bond strength data are shown in Table 1. There were statistically significant differences among different groups (p<0.0001). Microshear bond strength means recorded for UE and ET were significantly higher than that of DE. Percentage of fracture patterns recorded for each experimental condition are shown in Table 1. Adhesive and mixed fractures prevailed in all groups, and this outcome indicates that dentin/adhesive interfaces were tested at microshear level.

Discussion

The acid treatment selected in the current study to simulate enamel demineralization lesion is in compliance with parameters used in similar researches, which adopted a wide variety of methodologies, whose protocols included from the direct application of 30% phosphoric acid for 30 s (9,11) to holding samples in buffered acidic solution (pH 4.9, at 37 °C) for 18 days prior to enamel acid etching (5). Overall, the demineralization process depends on the tests to be applied to the enamel's structure: Quick white spot methods are adopted whenever enamel surface characterization is necessary, whereas slower methods are applied for subsurface characterization purposes. Enamel matrix proteins appear to be a viable solution to reverse incipient carious spots (5,10,11) and dental erosion (11,12), as well as in preventive procedures focused on improving enamel resistance to future acidic challenges (12,13). Based on some methodologies, amelogenin solutions are produced through the extraction of developing porcine teeth, protein and through protein purification (11) and production based on molecule expression by certain Escherichia Coli strains; or using commercial solutions such as Emdogain (5,12). Although tests were herein performed to characterize the surface of enamel samples, the research group in the current study has made the option to adopt a slow demineralization technique in order to mimic the white spot lesion as accurately as possible; besides, samples were prepared to be used in future tests focused on characterizing the enamel in subsurface layers. Emdogain was the solution adopted in the current study since it is substantiated by different studies available in the literature (5,12). Such decision was made to evaluate a standardized solution, which facilitates the reproducibility of tests carried out in this type of research (5,12).

It is important emphasizing the need of applying the protein solution right after the conditioning and washing procedures, since the native enamel matrix is a protein scaffold and, consequently, it is susceptible to denaturation or proteolysis, which makes it unfeasible for biomimetic use (15). Some researchers have drawn attention to the instability of amelogenin molecules under non-physiological pH and temperature conditions (1,7). There is great variation in the literature about the time the enamel matrix solution takes to enable the biomimetic formation of a scaffold susceptible to regeneration. Assessed times varied from 50 s (10) to 96 h (divided into two 48-h cycles) (12), depending on the solution. This time was selected based on the feasibility of enabling this procedure in a clinical situation of 15 min, since this time, in addition to the time necessary to properly isolate the area, is enough to perform the procedure in a supposed patient, as well as to enable the protein solution to form the new framework to be mineralized.

The remineralizing solution is responsible for providing the necessary ion bank for hydroxyapatite crystallization. Its components comprise mostly salts, which are dissociated in calcium, phosphate and hydroxyl ions in aqueous media. Other components such as fluorides are often added to the solution in order to mimic natural saliva. The available literature presents little divergence about the composition and concentrations of salts used to form such solutions. Variations of buffered artificial saliva formulations are often used at pH 7; however, there is no consensus about the enamel sample permanence time in this solution, which varies from 11 h (13) to 21 days (5), although most studies adopt permanence time of 7 days (11). The current study used artificial saliva solution (pH 7) as remineralizing solution, for 5 days. In addition to phosphate and calcium ions, the solution included other ions, which are often found in human saliva. Despite such choice, it was possible observing the hardness and the FT-IR spectrum that characterized the mineralized enamel in samples collected from the ET group.

The hardness of the demineralized enamel increased after the EMD treatment was applied to the samples in the current study. This outcome confirms the successful handling of enamel samples and, therefore, the successful remineralization of this dental structure. It is worth mentioning that this outcome was also

Table 1. Microshear bond strength means (MPa) and standard deviation (SD), and percentage of fracture patterns

Groups (n=10)	Mean (SD)	Fracture Patterns (%)		
		Adhesive	Mixed	Cohesive
UE	14.59 (1.25) A	85	10	5
DE	11.85 (0.70) B	80	5	15
ET	15.01 (0.90) A	80	10	10

Different letters indicate statistically significant differences between mean values, according to the Tukey's test, at 5% significance level. UE: Untouched Enamel; DE: Demineralized Enamel; ET: Demineralized Enamel Treated with EMD.

recorded in studies (5,10-13) that have presented very poorly-aligned methodologies, as well as variations in aspects such as acid challenge type, enamel conditioning protocol, protein solution composition and action time, remineralizing solution composition and remineralization time. Assumingly, there can be variations in the clinical use of these substances or such use should be further investigated to optimize the available techniques and maximize their results. The increase in ET group hardness after EMD treatment is a find that illustrates success on mounting a brand new protein scaffold over previously exposed enamel matrix that allowed mineral deposit under controlled pH, temperature and ions offer.

The FT-IR test in the region between 1200-900 cm^{-1}, which is compatible with the presence of phosphate (16), recorded broader and stronger signals for samples collected from the UE group, a fact that indicated higher incidence of phosphate ions that is a hydroxyapatite indicator. Values recorded for the ET group were very close to the ones recorded for the UE group. The DE group showed bands with similar shape, although they presented significantly lower intensity, a fact that indicated lower incidence of hydroxyapatite. FT-IR test results confirmed the mineralization degree in the controls (UE and DE groups) and showed that mineralization degree in the ET group was close to that of untouched enamel (UE), as corroborated by another study (10).

Soft hypomineralized enamels are porous and prone to fractures due to cavities and structural anomalies (17). Electron-microscopic examinations have shown that enamel crystals seen in the hypomineralization of molar and incisor teeth are not organized and present a small number of calcium and phosphate ions (17). The present study has found hypomineralized enamel in the DE group (Fig. 1). The bonding to enamel should be a concern, since the stability of the resin-bonded dentin (18) and effectiveness of marginal sealing (19) depends on the bonding to surrounding enamel (20). Adherence results in the current study did not show statistically significant difference between the UE and ET groups. Although few studies have used demineralized enamel as substrate in bond strength evaluations, composite bonding to demineralized enamel appears to be lower than to sound enamel (21), probably because demineralized enamel presents lower mineral content, higher surface porosity (22), widened intercrystalline spaces and, consequently, larger pore volume than sound enamel (23), a fact that leads to unsatisfactory etching patterns and to resin monomer infiltrations (21). This fact was observed in the present study, in which the ED group statistically differed from the other groups. This outcome could lead to the aforementioned concerns. The present study mostly found failures in bonding interfaces, which were followed by mixed failures and by lower incidence of cohesive failures, which was relevant for credibility of the study (24). Pretest failure was not observed, likely because the substrate (enamel) presented sufficient resistance, despite caries induction.

The remineralization process could be explored chemically with a mapping of elements, molecules and crystallographically. However, there is a report of this measurement being performed by FTIR analysis (10). Thus, the present study could present this fact as a limitation. Other limitations are the lack of information about regenerated enamel resistance to future demineralizations, as well as the lack of data about the application of this solution type in studies in vivo. Clinically, our results could be extrapolated to the application in brackets bonded or cementation of veneer with preparation in enamel, in which the enamel has undergone demineralization. For this reason, clinically oriented studies should be conducted to evaluate other remineralized enamel aspects. Moreover, EMD use in biological tissues for a few years, its proven biocompatibility, as well as successful enamel remineralization rates found in different studies are plausible arguments to justify future studies in vivo. Thus, the current results are consistent with the herein tested hypotheses because there was difference in chemical composition [1] and the microhardness [2], as well as in the bond strength of composites between demineralized enamel and demineralized enamel treated with EMD [3]. It was possible getting to the following conclusions by taking into consideration the limitations of a study conducted in vitro: EMD application to demineralized enamel seems to have remineralized the enamel with similar mineralization indicators, as well as to similar microhardness and bond strength of composites between untouched sound and demineralized enamel treated with EMD.

Resumo

Este estudo avaliou a composição química e microdureza do esmalte humano tratado com solução de Derivados da Matriz do Esmalte (EMD) e a resistência de união entre compósito e este esmalte. Trinta amostras de esmalte humano foram aleatoriamente divididas em três grupos: Esmalte Intocado (UE), Esmalte Desmineralizado (DE) e Esmalte Desmineralizado Tratado com EMD (ET). Os grupos DE e ET foram submetidos a desafio ácido e ET tratado com EMD (O EMD foi aplicado diretamente sobre esmalte condicionado e deixado por 15 minutos). Amostras de cada grupo (n = 4) tiveram composição química avaliada através de espectroscopia no infravermelho por transformada de Fourier com reflectância total atenuada (FTIR-ATR). A microdureza Knoop das amostras de esmalte de cada grupo (n=10) foi mensurada. Para a resistência ao microcisalhamento, as amostras foram condicionadas por 30 s, o adesivo aplicado e foto-ativado por 10 s. Duas matrizes plásticas (1 mm de comprimento) foram posicionadas sobre as amostras, preenchidas com compósito Filtek Z350 XT e foto-atiavadas por 20 s cada. As matrizes foram removidas e a resistência ao microcisalhamento de cada grupo (n=10) foi testada. Os dados foram submetidos ao teste de Kruskal-Wallis (para análise da microdureza), à análise de variância e ao teste de Tukey (para análise da resistência ao microcisalhamento); (α=0.05). Os resultados do FT-IR mostraram fosfato (indicador de hidroxiapatita) na banda entre 900-1200 cm^{-1} nos

grupos UE e ET, diferentemente do grupo DE. Análises de microdureza e microcisalhamento demonstraram resultados estatisticamente superiores para os grupos UE e ET quando comparados ao DE. A aplicação de EMD ao esmalte desmineralizado parece ter remineralizado o esmalte; assim, a microdureza e a resistência de união foram semelhantes entre os grupos UE e ET.

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