

Chemical exchange between dentin pre-treatment with CPP-ACP and GIC on demineralized dentin in deciduous molars

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Aim: To assess the effect of the chemical exchange between restorations using high-viscosity glass ionomer cement (GIC) and the casein phosphopeptide amorphous calcium phosphate CPP-ACP treatment on primary demineralized dentin by analyzing the hardness changes caused by calcium, phosphate and fluoride uptake.

Methods: 40 deciduous molars were selected and randomly assigned to four groups according to dentin pretreatment and dentin condition. Class I cavity preparations were performed in 40 sound primary molar samples, equally divided into groups G1 (sound dentin) and G2 (demineralized dentin). Sub-groups (n = 10) were set in order to aid in investigating the isolated GIC action or its association with CPP-ACP. This study was conducted *in vitro* and assessed the chemical exchange under two conditions, namely: sound and demineralized dentin (pH cycling); This *in vitro* study examined the mechanical and chemical exchange under two conditions – sound and demineralized dentin (pH cycling) – to simulate the mineral loss that occurs for the caries lesion. The 40 teeth first received a topical application of ACP-CPP and a restoration of high viscosity GIC. The 20 teeth assigned to the groups (G1 and G2) were only restored with GIC. The specimens were sliced and prepared for Knoop hardness test (KHN), Micro Raman, and FEG microhardness analysis groups. The statistical analysis used ANOVA and Bonferroni post-test at a 5% significance level. EDS (Dispersive Energy Spectroscopy) and FEG (High-resolution scanning electron microscope) data were qualitatively described.

Results: Increased hardness was observed in all sites that had direct contact with GIC in the sound and demineralized dentin samples in all groups ($p < 0.001$); microhardness showed no differences after CPP-ACP application ($p > 0.05$). The direct contact between GIC in sound and demineralized dentins resulted in an increased phosphate peak in the FEG and EDS evaluations.

Conclusion: ACP-CPP associated with GIC showed no increase in microhardness values of the demineralized dentin substrate. The exchange between the GIC and the demineralized dentin may induce changes in the mechanical properties of the substrate and in the uptake of mineral ions.

Uniterms: Dentin. Prevention. Caries. Remineralization. Demineralization. Biomechanics.

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INTRODUCTION

The scientific community today recommends minimal intervention principles in Dentistry. Deep carious lesions present remaining dentin with reduced thickness (less than 0.5 mm);

the treatment of choice in this case indicates the use of procedures capable of preserving tooth structure, such as partial caries removal¹. Thus, the use of biocompatible materials, which are applied to the dentin, allows and/or promotes tissue repair¹, as well as promotes the proper sealing of

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the tooth-restoration interface and decreases the possibility of microleakages². Studies on chemical exchanges between bioactive materials and hard dental tissues provide information about changes in the physicochemical properties of the involved dental structures³.

The casein protein derived from milk is referred to in the literature as a dental-tissue remineralizing agent and as a potential anticariogenic^{4,5}. Supersaturation stability in the oral environment promotes high calcium and phosphate ion levels in the tooth surface, and it allows the gradients of these ions to promote a remineralized dental structure.¹ The casein phosphopeptide (CPP) binds to the amorphous calcium phosphate (ACP) and forms the casein phosphopeptide amorphous calcium phosphate (CPP-ACP) complex^{4,5}, which is capable of donating calcium and phosphate ions to the tooth structure through nanoparticles, thus forming the dental structure⁵. Some studies indicate that the calcium and phosphate ions are released into the dental plaques, in a supersaturated state, in turn promoting the remineralization process. These studies defend that CPP-ACP can act as an ion reservoir when the dental tissues are supersaturated^{4,5}.

Remineralization technology, based on calcium phosphate, is a promising alternative non-invasive technique to caries lesion management^{5,6}. The research for a biomaterial capable of improving the chemical and mechanical properties of the demineralized dental substrate from the chemical interaction with dentin was proposed in this study by including CPP-ACP to increase the amount of available calcium in an attempt to alkalize the demineralized dentin and to seal this dentin through the atraumatic restoration technique. Therefore, this *in vitro* study aimed to evaluate the chemical exchange of CPP-ACP applied prior to restoration of the GIC in deep demineralized deciduous dentin and repercussions on the mechanical and chemical properties of the deciduous dentin.

MATERIAL AND METHODS

The present research was approved by the Research Ethics Committee from the State University of Ponta Grossa, logged under protocol number 1.565.693.

SAMPLE

This research was performed with sound human primary molars (n = 40), which were collected in accordance with the requirements of the Human Research Ethics Committee of the State University of Ponta Grossa. This sample consisted of 40 primary molars showing no crown defects or carious lesions. The remaining root portions were removed, using a high-speed diamond bur (2121 KG Sorensen®, Cotia, SP, Brazil), and the teeth were stored in distilled water to avoid dehydration until cavity preparation.

STUDY DESIGN

Knoop hardness, EDS, and FEG tests were used to evaluate the following dentin conditions: sound/demineralized, treated or not with CPP-ACP paste, with/without contact with GIC. For this purpose, class I cavity preparations were performed on 40 selected primary molars. The teeth were divided into 2 groups (n = 20) according to the dentin condition: sound (G1) and demineralized (G2). Subgroups (n = 10) were formed to analyze the GIC's isolated actions and their actions in combination with CPP-ACP.

The cavities in all groups were divided in halves, and one-half of each sample was isolated with nail varnish to create the control areas in each group (Figure 1). All cavities were restored with high-viscosity glass ionomer cement, but only G1CPP-ACP and G2CPP-ACP received the CPP-ACP pretreatment before restoration (Figure 1).

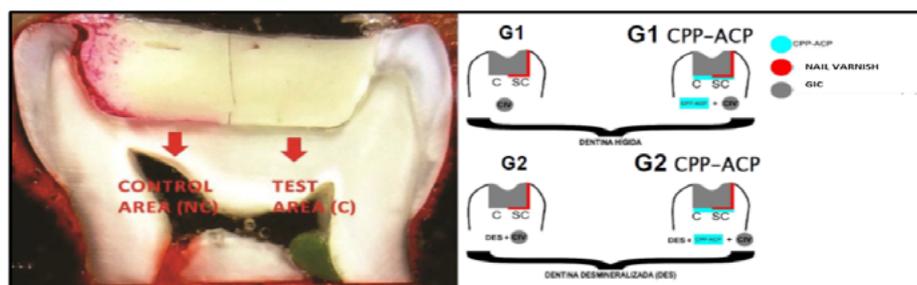


Figure 1 - Sagittal section of teeth restored with GIC in two dentin conditions: sound and demineralized (G1, G2), and with topical CPP-ACP paste application before restoration (G1CPP-ACP, G2CPP-ACP). A red nail varnish layer was applied to avoid the direct contact of GIC with half of the dentin-control area (NC) and with the tested area (C).

PREPARATION OF THE CLASS I CAVITIES

Cavity preparations started in the center of the occlusal surface of each primary molar. The cavity size was based on a deep cavity, characteristic of deep carious lesions. Deep cavities are considered to be those with more than 0.5 mm and less than 1 mm of remaining structure between the floor and the pulp. Cavity preparation began in the center of the occlusal surface of each primary molar. The visual examination through a stereoscopic magnifying glass (10X) was performed to assess the specimens, allowing on to check whether or not the pulp chamber was exposed due to cavity preparation; if so, the tooth was discarded.

PROCEDURES FOR PH CYCLING

The teeth in G2 (n = 20) were subjected to dentin demineralization to simulate carious lesions through the pH cycling method, according to the established protocol¹¹.

Artificial dentine carious lesions were created by a pH cycle procedure, in which each specimen was immersed for 8 h in a demineralizing solution and for 16 h in a remineralizing solution. The cycle was repeated for 14 consecutive days at room temperature. The solutions used in pH cycling were manipulated in the Pharmaceutical Science Laboratory at State University of Ponta Grossa. The formulations⁷ were as follow: demineralizing solution (pH 4.8) containing 2.2 mM calcium chloride (CaCl₂); 2.2 mM phosphate Sodium (Na₂PO₄) and 50 mM acetic acid; and remineralizing solution (pH 7.0) containing 1.5 mM calcium chloride (CaCl₂), 0.9 mM phosphate Sodium (Na₂PO₄), and 0.15 mM potassium chloride (KCl).

APPLICATION OF CPP-ACP

Before the restorative procedures, all the cavities received a layer of nail varnish on the mesial side cavity. This research design stipulated the division of the cavity into two sites: control area (C) and test area (T). For these groups, after the application of the Ketac polyacrylic acid conditioner (3M ESPE, St. Paul, MN, USA), but before the restoration, a pretreatment with CPP-ACP was applied on the dentin for 1 min with the aid of a GIC dispenser, and the volume to each cavity was standardized. GIC was taken to the cavity with the aid of a measuring spoon. After, CPP-ACP was not removed.

RESTORATIVE PROCEDURES

All cavities were filled with high-viscosity GIC (Ketac Molar Easymix[®], 3M ESPE, St. Paul, MN, USA). The cavities were treated with liquid Ketac (3M ESPE, St. Paul, MN, USA) for 10s, washed with air/water spray for 20s, dried with a gentle dry compressed air stream and immediately filled with the GIC. The GIC was dosed at ratio 2:2 (powder and liquid) and manipulated on the block using a plastic spatula (Duflex[®], Rio de Janeiro, RJ, Brazil). The mixture was inserted with an applicator syringe (Precision Maquira[®], Maringa, PR, Brazil) until the cavity was completely filled. GIC was left to rest for 3 min in accordance with manufacturer instructions. The teeth were then stored in a humidifier for 24 h at 37°C.

PREPARING THE SPECIMENS FOR TESTING

The teeth were fixed in a cutting machine (Isomet[®] 1000 Precision Saw Buehler, Lake Bluff, IL, USA) and vertically sectioned using a diamond disk (1.3 mm Precision Saw, Lake Bluff IL[®], USA) at 300 rpm to produce dental slices (n = 3) of approximately 1.1 mm in thickness. Two dental slices were mounted in the center of PVC cylinders (12x20 mm) (Tiger[®], Joinville, SC, Brazil), which were attached to a glass plate using a double-sided tape (3M[®], SUMARE, SP, Brazil). The cylinders were filled with colorless acrylic resin (JET[®], Clear Field, SP, Brazil) made using the powder and liquid technique. Finally, they were stored at 37°C in 100% humidity environment at 37°C for 24 h.

MICROHARDNESS TEST

The microhardness test was performed in a microhardness apparatus (Shimadzu[®], Kyoto, Japan) with Knoop indenter. The following applied loads were used for 30s: 25g for sound dentin, and 10g for demineralized dentin⁷. The loads were applied 50 µm below the specimens' cavity floor. The mean between these three measures was considered the microhardness value of the specimen.

DISPERSIVE ENERGY SPECTROSCOPY TEST (EDS) AND HIGH-RESOLUTION SCANNING ELECTRON MICROSCOPE (SEM)

The ions in the dentin were assessed through energy dispersive spectroscopy (EDS) in a scanning electron microscope (SEM) (Mira 3, Tescan, Kohoutovice, Česká republika) for

field effect. Images and the morphological characterization were obtained for FEG analysis. The reading was performed at a 50 micron depth of the cavity base, with magnification of 5000X. The areas of measure were divided in areas with contact (C) and without contact (NC) between the restorative material and the dentin.

STATISTICAL ANALYSIS

The normality distribution was assessed through the D'Agostino & Pearson, and Shapiro Wilk tests; variance homogeneity was tested through the Levene's test. The hardness and EDS data were analyzed through ANOVA (2 criteria) by taking into consideration such factors as dentin (sound and demineralized), treatment (with and without CPP-ACP), and treatment-dentin interaction. The 5% significance level ($\alpha = 0.05$) was adopted. The FEG data from each group were qualitatively analyzed. The statistical software GraphPad Prism, version 5 for Windows, (GraphPad Software, San Diego California, USA) was used for hardness-data analysis.

RESULTS

There was a significant difference between hardness values in the treatment using MiPaste™ in areas SC and C ($p < 0.0001$). Significant difference in the dentin factor was found in areas NC ($p < 0.0001$) and C ($p < 0.0001$). There was no significant difference between the dentin-treatment interaction in NC ($p = 0.203$) and C ($p = 0.166$) (Figure 2). The EDS mineral assessment showed the Ca, P, and F ion percentages. Significant difference was found in the P values ($p = 0.0001$) of the NC areas. There was no significant difference in the types of dentin ($p = 0.218$), treatment ($p = 0.077$), and interactions ($p > 0.05$). There was no significant difference between treatments ($p = 0.865$) and interactions ($p > 0.05$) in the Contact areas. A significant difference between the types of dentin ($p = 0.027$) and minerals ($p < 0.0001$) was observed. The ionic exchange between GIC and the dentine pre-treated with the CPP-ACP paste promoted significant changes in the phosphorus mineral (Figure 3).

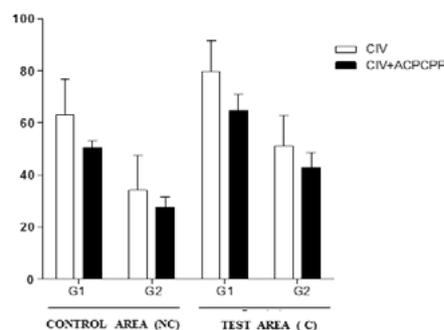


Figure 2 - Mean and standard deviation of the Knoop hardness values found in the sound (G1) and demineralized (G2) dentins, with and without CPP- ACP treatment in the areas without (NC) and with contact (C) to the restorative material (GIC). (*) Significant difference in the hardness values found in treatment using MiPaste™ in the NC and C areas ($p < 0.0001$). (*) Significant difference in the dentin factor in NC ($p < 0.0001$) and C areas ($p < 0.0001$). No significant difference in the interaction (dentin and treatment factors), according to the NC ($p = 0.203$) and C ($p = 0.166$) areas. ANOVA (with two criteria) and Bonferroni post-test.

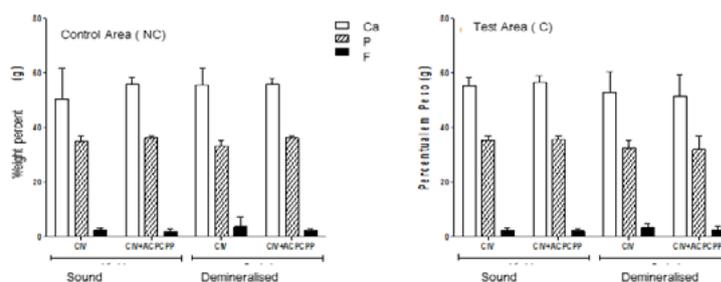


Figure 3 - Mean and standard deviation of the weight (g) percentage shown by calcium, phosphorus, and fluoride in sound and demineralized dentins, with and without contact to the glass ionomer material (GIC), and with the ionomer + bioavailable casein-phosphate paste (CIV + CPP-ACP). No-contact area: non-significant differences between the types of dentin ($p = 0.218$), treatment ($p = 0.077$), and interactions ($p > 0.05$); significant difference between minerals ($p < 0.0001$). Contact: non-significant difference between treatments ($p = 0.865$) and interactions ($p > 0.05$); significant difference between the types of dentin ($p = 0.027$) and minerals ($p < 0.0001$). ANOVA (with two criteria) and Bonferroni post-test.

The images of the sound and demineralized dentin treated with CPP-ACP and GIC were obtained through FEG (Figure 4). Regular mineral deposits in the dentin treated with CPP-ACP and the obliteration of the dentinal tubules were noticed in treatments (A) and (C).

The presence of surface irregularities in the sound dentin treated with the direct placement of GIC was noticed in B. The demineralized carious dentin surface without restorative-material adhesion with open dentinal tubules was observed in treatment (D).

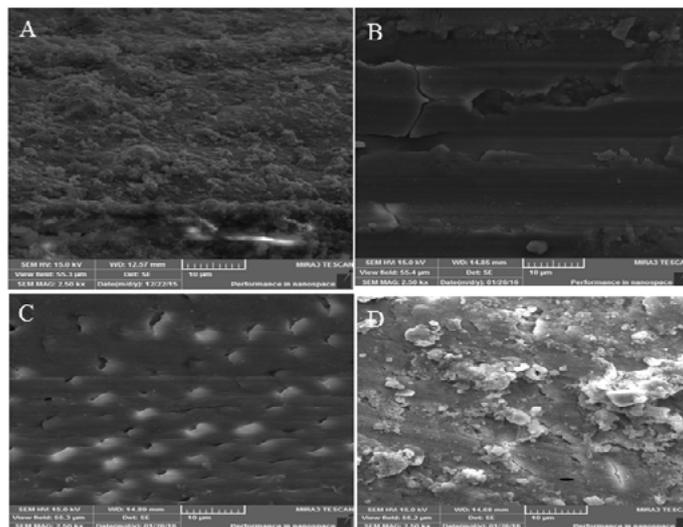


Figure 4 - Images from FEG. (A): Sound dentin. (B): Sound dentine treated with CPP-ACP and GIC. (C) Demineralized dentin without treatment. (D): demineralized dentin treated with CPP-ACP/GIC.

DISCUSSION

Despite the advances in dental care in recent decades, the knowledge about the physical and mechanical properties of the dental tissue allows one to report the clinical behaviors of restoration, although dental caries still affect many children. Among these physical and mechanical properties, the elasticity, resistance, and hardness modules are the most relevant^{2,8}. The specific treatment recommended for deciduous dentin, either by clinical or therapeutic differences in the dentin surface, can potentially affect clinical practices when they are compared to the dentin of the permanent teeth⁹. The chemical composition of primary teeth differs from that of permanent teeth, because it presents lesser thickness than the enamel and dentin of permanent teeth^{9,10}. The Ca and P percentage is lower in the primary teeth when compared to the permanent teeth^{10,11}.

The simulation of carious lesions dynamics *in vitro* has been the method applied to experiments assessing the materials supposed to be in contact with the carious dentin^{7,12}. The demineralized dentins were collected in artificial caries lesions generated through the pH cycling method^{7,10} in an attempt to simulate the dynamics of the mineral pH saturation interval associated with the natural caries formation process. This

is an appropriate laboratory method to assess the mineral losses and gains in the substrate.^{7,14} Marquezan et al¹⁰ found mineral losses in specimens presenting significant hardness reduction (34KHN). Some studies assessed the levels of ion exchange between the dentin and the restorative material through artificial caries lesions and concluded that the method conducted *in vitro* allows detailed studies about the interactions between restorative materials and the demineralized dentin^{3,9}. The conditions used in artificial caries lesions follow a similar pattern to simulate *in vivo* conditions; the artificial caries lesion presents an intact surface layer that is rich in minerals and a subsurface layer with low mineral content, a fact that brings the *in vitro* findings closer to the *in vivo* clinical situations.

The comparative analysis applied to the mechanical properties of the dentin has indicated that the central area of the coronal sound dentin has stronger hardness in permanent teeth than in deciduous teeth⁷. Several microhardness studies have shown that the enamel presents higher values than the dentin^{7,13} because its weight is comprised of approximately 70% minerals, 18% organic material, and 12% water^{7,13}. There is great difficulty in comparing the microhardness dentine values in different studies, due to the adoption of different test types used to set the mechanical properties.

The mineral losses or gains in dentin can be measured through spectroscopy and hardness tests^{16,17}.

The use of GIC as a restorative material is based on its properties, which are biologically favorable to fluoride release and clinical performance²³. Some studies have shown that calcium, phosphorus, and fluorine ions are released from GIC restorations in enamel and dentin, particularly in demineralized dentin; therefore, the present results reinforced the importance of using GIC as the material of choice for minimally invasive restorations.

Casein can serve as an ion reservoir during cariogenic attack^{7,8} by maintaining the free calcium and phosphate ion supersaturation state in the substrate, and by decreasing and increasing demineralization to result in a tissue that is more resistant to acid attacks. The caries prevention strategies have been based on the direct application of substances such as CPP-ACP into the dentin in order to increase dentine resistance to cariogenic attacks. The good clinical acceptance of CPP-ACP results from ACP's ability to release calcium and phosphate ions in the acid oral environment so that ions can remineralize the treated dental tissue²¹⁻²³.

Different methods, such as energy dispersive spectrometry (EDS), wavelength fluorescence scatter X-rays spectroscopy (EDXRF), atomic absorption spectrophotometry (AAS), and FT-Raman, can be used to assess the mineral content of the dental hard tissue¹¹. However, the techniques used in studies (EDS and FEG) can assess multiple elements at the same time^{11,12}. The EDS and FEG methods were used to assess the changes in dentin and allowed for the recording of the calcium, phosphorus, and fluorine ion concentration in the surface of the dentin treated through microstructural and morphological analysis^{10,11}. The EDS showed significant improvement in the mineral properties of the dentin, mainly in phosphorus ions, both in the sound and the demineralized dentin treated with Mi Paste in combination with GIC. Although the remineralization studies based on mineral uptake are insufficient to assess the success of contemporary remineralization strategies. The present study examined the quantification or the capture of ions after 24 hours of application. This was performed to measure the electroless deposition, as it enables one to improve the mechanical properties of dentine in the interface, consequently favoring the lower degradation and clinical restoration performance. Therefore, this procedure was able to aid in predicting the validity of the proposal.

Dentin is composed of organic and inorganic components; the primary inorganic components of the dental hard tissue are calcium (Ca) and phosphorus (P), which are found in hydroxyapatite crystals¹³. It has been reported that the Ca/P ratio in dentin depends on such factors as the hydroxyapatite crystal type, Ca, and anatomical location^{12,13}. Moreover, it has been reported that by changing the Ca/P ratio in the dentin, it is possible to change the morphology of the dentin and affecting the adhesion of restorative dental materials^{10,11,13,14}. According to the present results, the Ca/P ratio in the dentin of deciduous teeth was affected by the pretreatment with Mi Paste. The results showed that this treatment has validated mineral EDS alterations because of the obtained Ca, P, and F ion percentages. The ion exchange between Mi Paste and dental tissue in combination with GIC, as well as statistically significant P ion values, were observed. Despite the instability and the non-significant differences, the CPP-ACP reactivity should be further explored to assess accession and bioactivity in addition to the improvement of the absorption properties affecting the mechanical and chemical properties of the tissue treated in this study

The present results evidenced that the pretreatment with Mi Paste validated the change in hardness values due to significant differences found in the areas with and without MiPaste™ contact. Accordingly, the application of CPP-ACP paste could promote a preventive effect, increase remineralization and demineralization, and decrease dentin susceptibility^{5-7,16}. The CPP-ACP pretreatment provided all the elements needed to remineralize the tooth surface and the biofilm¹¹, as it provides soluble calcium, fluoride, and phosphate to generate fluorapatite remineralization, which is more resistant to future etching¹⁵⁻¹⁷. Lastly, a lack of sample calculations represents a potential limitation of this study. However, this does not diminish the importance of the information on the use of CPP-ACP as a promising biomaterial of dentinal mineralization in a sealed carious cavity.

Although the literature has several studies showing the interaction of dental materials with enamel^{18,19}, few studies have assessed remineralization in the deciduous dentin substrate^{20,21}. The presence of CPP-ACP on the dentin surface led to decreased demineralization and to increased remineralization, in comparison to the dentin surfaces without this agent. This suggests that the nanocomplexes formed with CPP-ACP could protect the adjacent tooth tissue

from pH changes by reducing demineralization, as well as aid in remineralization, which would lead some researchers to incorporate this material into dental products such as GIC²⁰⁻²². Research has shown that the release of inorganic phosphate from CPP-ACP, and fluoride sample containing CPP-ACP/GIC, have promoted greater protection to the adjacent dentin during acid challenge²².

The Mi Paste application in the dentin before GIC application to fill the cavity showed mineral and phosphorus ion gains. Despite the instability and non-significant differences, CPP-ACP reactivity should be further explored to assess accession and bioactivity, in addition to the improvement in absorption properties affecting the mechanical and chemical properties of the treated tissue.

CONCLUSION

From the results of this study, CPP-ACP could be an alternative dental material for deep carious lesions in deciduous teeth, due to changes in the chemical and mechanical properties of the demineralized deciduous dentin. The increase in dentin hardness and changes in mineral contents were due to ion exchanges from GIC to dentin. More studies on the effect of CPP-ACP on demineralized dentin should be performed.

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Mudanças químicas entre dentina pré-tratada com CPP-ACP e CIV na dentina desmineralizada de molares decíduos.

Objetivo: Avaliar o efeito das trocas químicas entre restaurações de cimento de ionômero de vidro de alta viscosidade (GIC) e CPP-ACP para o tratamento da dentina decídua desmineralizada por meio de análise em mudanças químicas causadas pelo cálcio, fosfato e fluoreto.

Métodos: 40 molares decíduos foram selecionadas e aleatoriamente divididos em quatro grupos de acordo com o pré-tratamento e condição dentinária. Cavidades Classe I foram preparadas em 40 molares decíduos, igualmente divididos em grupo G1 (dentina hígida) e G2 (dentina desmineralizada). Subgrupos (n = 10) foram formados para investigar a ação isolada do GIC ou associado com CPP-ACP. O estudo foi realizado in vitro e avaliado as trocas químicas sob duas condições: dentina hígida e desmineralizada (ciclagem de pH); Este estudo in vitro avaliou as mudanças químicas e mecânicas sob duas condições: dentina hígida e desmineralizada (ciclagem de pH) para simular as perdas minerais decorrentes das lesões cáries. Os 40 dentes (G1DMT, G2DMT) receberam primeira aplicação de ACP-CPP e restauração com CIV de alta viscosidade. Os 20 dentes foram atribuídos aos grupos (G1 e G2) foram restaurados com GIC. Os espécimes foram fatiados e preparados para o teste de dureza Knoop (KHN), Micro Raman and FEG análise de microdureza. A análise estatística utilizou ANOVA e Bonferroni post-test com nível de significância de 5%. EDS (Espectroscopia de Energia Dispersiva) e FEG (Microscopia de alta resolução de elétrons de varredura) foram qualitativamente descritos.

Resultados: Aumento da dureza foi observado em todos os sítios em contato direto com CIV em amostras de dentinas hígida e desmineralizada em todos os grupos. ($p < 0,001$); a microdureza mostrou diferenças não-significativas com aplicação de CPP-ACP ($p > 0,05$). O contato direto entre GIC em dentinas hígida e desmineralizada resultou em aumento do pico de fosfato nas avaliações de FEG EDS.

Conclusão: ACP-CPP associado ao GIC não resultou em aumento de valores de microdureza no substrato dentinário desmineralizado. As mudanças entre GIC e dentina desmineralizada podem induzir mudanças nas propriedades mecânicas do substrato e aumentar a incorporação de íons minerais.

Descritores: Dentina. Prevenção. Cárie. Remineralização. Desmineralização. Biomecânica.