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Original Research

Effectiveness of three biomimetic remineralization procedures in artificial caries-affected dentin – an in-vitro pilot study

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ABSTRACT

Objectives: Biomimetic remineralization strategies offer promising paths for managing caries-affected dentin by restoring its morphology and structural integrity. This pilot study aimed to assess the morphological and structural changes in dentin following two chemical artificial caries-affected dentin (ACAD) protocols, evaluate the effects of three biomimetic remineralization procedures (BRPs) on dentin structure, and determine the most effective ACAD-BRP combination. The BRPs studied were casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), self-assembling P11-4 (SAP-P11-4), and calcium phosphate polymer-induced liquid precursor (Ca/P-PILP).

Methods: Seventy-two specimens of healthy human dentin were divided into twelve groups. Half of each group's samples were analyzed using field-emission gun environmental scanning electron microscope/energy dispersive X-ray, while the other half were analyzed by X-ray diffraction and micro-Raman spectroscopy.

Results: Results demonstrate alterations in dentin morphology and structure induced by the ACAD protocols, affirming their effectiveness in mimicking caries-affected dentin. Among the BRPs evaluated, CPP-ACP emerged as the most promising agent for promoting remineralization and restoring dentin structure. The combination of pH cycling with CPP-ACP exhibited superior efficacy in promoting dentin remineralization compared to other combinations.

Conclusions: These findings highlight the potential of biomimetic remineralization strategies, particularly CPP-ACP combined with pH cycling, as effective approaches for managing caries-affected dentin and preserving dental health. (Rev Port Estomatol Med Dent Cir Maxilofac. 2024;65(4):188-196)

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Eficácia de três procedimentos de remineralização biomimética na dentina afetada por cárie – estudo piloto *in vitro*

Palavras-chave:
Biomimética
Matriz de dentina desmineralizada
Dentina
Remineralização dentária

R E S U M O

Objetivos: As estratégias de remineralização biomimética oferecem caminhos promissores para a gestão da dentina afetada por cárie, restaurando a morfologia e integridade estrutural. Este estudo piloto teve como objetivo avaliar as alterações morfológicas e estruturais na dentina após dois protocolos químicos de dentina artificialmente afetada por cárie (DAAC), avaliar os efeitos de três procedimentos de remineralização biomimética (PRB) na estrutura da dentina e determinar a combinação mais eficaz de protocolo de DAAC e PRB. Os PRB estudados foram fosfopeptídeo de caseína-fosfato de cálcio amorfo (CPP-ACP), péptido de automontagem P11-4 (SAP-P11-4) e o indutor do polímero precursor líquido do fosfato de cálcio (Ca/P-PILP).

Métodos: Dividiram-se 72 amostras de dentina humana saudável em doze grupos. Metade das amostras de cada grupo foram analisadas por microscópio eletrónico de varrimento/raios X de dispersão de energia, enquanto a outra metade foi analisada por difração de raios X e espectroscopia de micro Raman.

Resultados: Os resultados demonstram alterações na morfologia e estrutura da dentina induzidas pelos protocolos DAAC, confirmando a sua eficácia em mimetizar esta dentina. Entre os PRB avaliados, o CPP-ACP emergiu como o agente mais promissor para promover a remineralização e restaurar a estrutura da dentina. A combinação de ciclos de pH com CPP-ACP exibiu eficácia superior na promoção da remineralização dentinária comparativamente a outras combinações.

Conclusões: Estes resultados sublinham o potencial das estratégias de remineralização biomimética, particularmente CPP-ACP com os ciclos de pH, como abordagens eficazes para o tratamento da dentina afetada pela cárie e para a preservação da saúde dentária. (Rev Port Estomatol Med Dent Cir Maxilofac. 2024;65(4):188-196)

Introduction

Dental caries is a disease characterized by the progressive demineralization of dental tissues due to the action of acids produced by bacteria in the oral biofilm.[1] While affected enamel may be able to remineralize, dentin is often irreversibly damaged due to the breakdown of collagen bonds and the denaturation of odontoblasts.[2]

One objective of modern dentistry is to manage caries lesions non-invasively through remineralization, intended to prevent the progression of this disease and improve the resistance, aesthetics, and function of the affected tissues.[3] Biomimetic remineralization emerges as a promising response aimed at protecting exposed collagen in demineralized dentin layers by depositing minerals inside and between the fibers, as it occurs naturally, thus restoring structure and mechanical characteristics.[4] Remineralizing solutions containing calcium and phosphate have been used to promote the formation of a new remineralized layer on the dentin surface.[5-7]

Among the various biomimetic remineralization procedures (BRP), casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has stood out for its efficacy in promoting dentin remineralization.[5,8] CPP-ACP, a nano-complex of casein protein and amorphous calcium phosphate (ACP), releases and stabilizes high calcium and phosphate ion concentrations on tooth surfaces.[9] Self-assembling P_{11}-4 (SAP-P_{11}-4) is another promising agent that promotes remineralization by forming 3D hierarchical structures. SAP-P_{11}-4 binds to calcium ions, causing the precipitation of calcium phosphate salts in the structure, thus facilitating hydroxyapatite nucleation.[10] Another approach is the calcium phosphate polymer-induced liquid precursor (Ca/P-PILP), which has shown the potential to effectively promote demineralized dentin remineralization through analogs of non-collagenous proteins.[11]

Biomimetic remineralization is an important method for restoring the integrity of caries-affected dentin, and different options are available. Thus, it would be interesting to check in vitro the effectiveness of biomimetic remineralization with different agents in restoring the integrity of caries-affected dentin previously prepared with different chemical artificial caries-affected dentin (ACAD) protocols.

For that purpose, a pilot study was held with the following objectives: 1) to assess whether there are morphological and structural changes in dentin after two different chemical ACAD protocols; 2) to assess the effects of three different BRPs (CPP-ACP, SAP-P_{11}-4, and Ca/P-PILP) on the dentin structure; 3) to determine the most favorable ACAD-BRP combination. The null hypothesis is that there are no differences in any of these

objectives between ACAD and natural dentin. We will use advanced techniques, including field-emission gun environmental scanning electron microscope/energy-dispersive X-ray (FEG ESEM/EDAX), X-ray diffraction (XRD), and micro-Raman spectroscopy (MRS), to analyze the null hypothesis.

Material and Methods

The study was approved by the FMDUP Ethics Committee with the reference "Projeto nº 22/2021." For this pilot in-vitro study, 18 non-carious permanent human molars extracted for orthodontic or periodontal reasons were selected after clinical examination. The teeth with visible hypoplastic lesions, crack lines, or detectable carious defects were excluded from the study sample. The teeth were disinfected with 0.5% chloramine-T solution (Merck/Germany) for a week and stored in saline solution at 4°C until the experimental period (ISO/TS 11405) for up to 6 months after the extraction.

Eighteen dentin disks of 1.5-mm thickness were prepared using a hard-tissue microtome (Struers Accutom-5, United States of America (USA)) under running water. The exposed surface was polished with 120-grit silicon carbide sandpaper under running water (Struers Rotopol-11, USA) for 1 minute to simulate the smear layer.[5]

The dentin disks were then divided into four segments using a Horico diamond disk (Transvident, Germany) under running water. Seventy-two specimens were randomly distributed into 12 groups (Table 1) of N=6: one control group (G1) and 11 intervention groups (G2–G12). The control group in our study consisted of natural dentin, which was used as a baseline to compare the morphological and structural changes observed in the experimental groups. A single operator carried out all protocol procedures (Figure 1) by following the laboratory protocols for each group (Table 2). All groups were then analyzed using FEG ESEM/EDAX, MRS, and XRD.

Table 1. Experimental groups

Group	ACAD	BRP
1	Sound dentin	ø
2	CMC	ø
3	pH cycling	ø
4	Sound dentin	CPP-ACP
5	Sound dentin	SAP-P_{11}-4
6	Sound dentin	Ca/P-PILP
7	CMC	CPP-ACP
8	CMC	SAP-P_{11}-4
9	CMC	Ca/P-PILP
10	pH cycling	CPP-ACP
11	pH cycling	SAP-P_{11}-4
12	pH cycling	Ca/P-PILP

ACAD – artificially caries-affected dentin; BRP – biomimetic remineralization procedures; ø – absent; CMC – 6% carboxymethylcellulose acid gel; CPP-ACP – casein phosphopeptide-amorphous calcium phosphate; SAP-P_{11}-4 – self-assembling peptide$_{11}$-4; Ca/P-PILP – calcium phosphate polymer-induced liquid precursor.

Three samples were used for the FEG ESEM/EDAX analysis. A small groove was created in each specimen with a diamond disk before fixation in 3% glutaraldehyde for 24h at 4°C. They were then subjected to three consecutive 20-minute baths in sodium cacodylate solution, dehydrated in increasing concentrations of ethyl alcohol (Merck, Germany), and dried with crit-

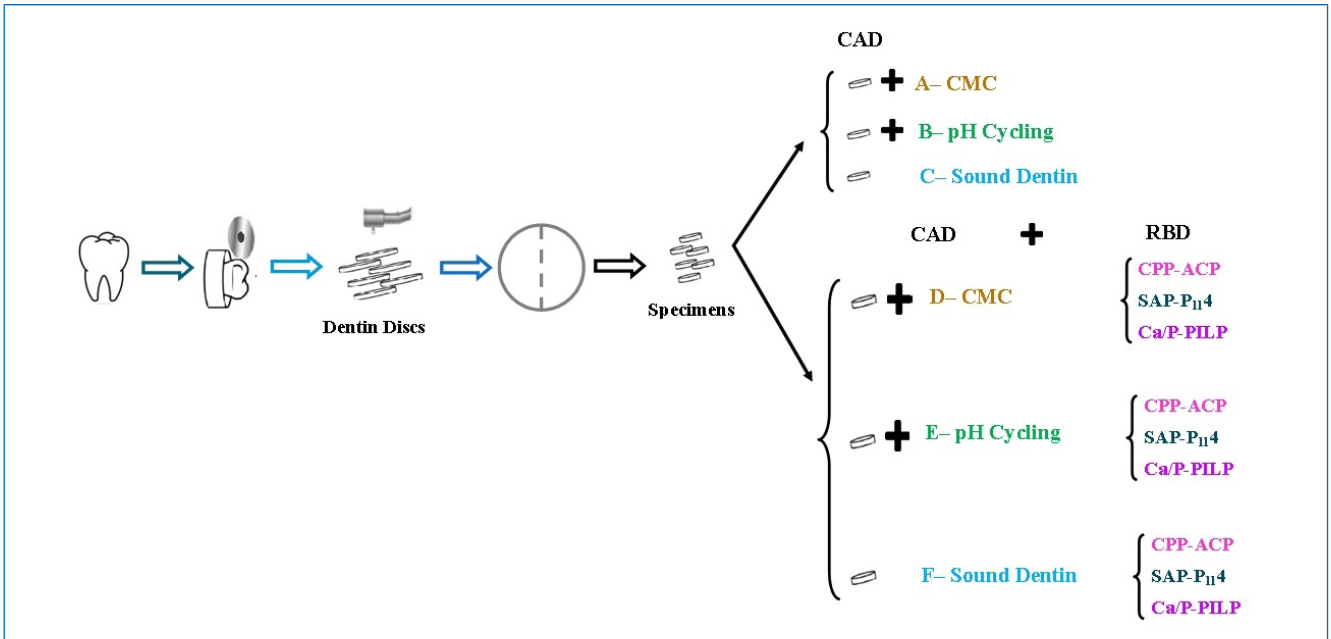

Figure 1. Experimental protocol

Table 2. Description of laboratory protocols

Solution	Preparation	Application
CMC	0.1 M lactic acid (Merck, Germany) titrated with a potassium hydroxide solution (Sigma-Aldrich, USA) at pH=5.0[27]	The specimens were immersed in 0.4 g of CMC (Thermo Scientific, USA) for 48 h (hours) at 37°C without renewal.[27]
Demineralizing pH cycling	50 mM acetic acid (Merck, Germany), 2.2 mM calcium chloride (Enzymatic, USA), and 2.2 mM monosodium phosphate (Sigma-Aldrich, USA), at pH=4.8[18]	Each specimen was subjected to 24-h cycles of 8 h immersed in a 10-mL demineralization solution followed by 16 h immersed in a 10-mL remineralization solution for 15 days with constant renewal.[18]
Remineralizing pH cycling	1.5 mM calcium chloride and 0.9 mM monopotassium phosphate (Sigma-Aldrich, USA), at pH=7.0[18]	
CPP-ACP	Tooth Mousse GC, Japan	100 µL were applied to the sample's surface and rubbed with a microbrush for 1 min. The surface was rinsed with distilled water for 10 s (seconds).[25]
SAP-P_{11}-4	Credentis, Switzerland	50 µL were applied to the sample's surface with the applicator for 5 min.[27] It was then submerged for 2 min in 10 mL of Dulbecco's phosphate-buffered saline (Thermo Scientific, USA) at pH=7.4.
Ca-P/PILP	Overnight, at room temperature and on a magnetic mixer, 2 mL of a 0.1-M sodium phosphate solution (Fisher Scientific, USA) was mixed with a 0.4-mL solution of 0.3-g/ml polyacrylic acid (Sigma- Aldrich, USA) and 0.15-g/mL polyaspartic acid (Thermo Scientific, USA). In the morning, 2 mL of a solution containing 0.2 mL of 1-M calcium chloride and 0.2 mL of 0.3-g/ml polyaspartic acid was vigorously stirred until diluted using a magnetic mixer at room temperature. 2 mL of the nighttime solution was added vigorously to 0.2 mL of the daytime solution, adjusting the pH to 7.4.[18]	Each specimen was submerged in 5 mL of the solution for 20 days at 37°C with constant stirring and renewed every 5 days.[18]
3% glutaraldehyde	Buffered solution (Sigma-Aldrich, USA) with 0.2-M sodium cacodylate (Thermo Scientific, USA) at pH=7.2	Fixation for 24 h at 4°C

ical point drying.[12] Afterward, the samples were briefly submerged in liquid nitrogen and divided into two fragments by applying pressure to create the cross-section view. These fragments were fixed to SEM stubs with carbon tape and metalized with a thin gold/palladium film by sputtering using the SPI Module Sputter Coater. A blind operator conducted the FEG ESEM/EDAX examination (FEI Quanta 400/Genesis X4M). Qualitative and quantitative analyses were performed: qualitative analysis of microphotographs obtained in FEG ESEM and quantitative analysis based on the average of three specimen measurements obtained by a software coupled with EDAX.

The remaining three samples were analyzed using MRS and XRD. The MRS analysis was conducted using the inVia Raman Microscope (Renishaw, United Kingdom) equipped with a 785-nm red diode laser. Measurements were carried out at room temperature, focusing on three different points on the specimens with a 50x magnifying lens. Acquisition parameters included a range of 200-1800cm^{-1}, an integration time of 10s per scan, the addition of three scans, and a maximum laser power of 115 mW to avoid heat-induced artifacts. Spectra were presented and compared after subtraction of a smoothed baseline and normalization to percentage values using the peak of the most intense Raman mode at around 960 cm^{-1}. Raman mode parameters were calculated using IgorPro software based on the best fit of a sum of damped oscillator functions.

For the XRD analysis, the same samples were ground, prepared, and mounted on a standard sample holder for powders. Measurements were conducted at room temperature using a PANalytical X'Pert Pro diffractometer equipped with an X'Celerator detector and a secondary monochromator in θ/2θ Bragg-Brentano geometry. The measurements used were 40 kV and 30 mA, Cu Kα radiation (λα1 = 1.54060 Å; λα2 = 1.54443 Å), 0.017°/step, 100 s/step, and 2θ 10-90° angular range. Diffractograms were interpreted (qualitative evaluation), phases were identified using HighScore Plus 4.8 software, and Rietveld refinement of the diffractograms was conducted using the PowderCell 2.4 software. A quantitative analysis using a simple average of three samples was executed to analyze MRS comparative modes and XRD Rietveld analysis.

Results

In G2, the FEG ESEM/EDAX analysis revealed no noteworthy changes in inorganic ion concentrations and only slight exposure of the collagen layer within the dentinal tubules. In G7, G8, and G9, where BRPs were performed, no ion concentration variation was observed either. On the other hand, G3 showed a clear change in dentinal tubules—they were widely open and more porous, resulting in demineralization evidenced by decreased levels of inorganic ions calcium and phosphate. G4 had a slight change in the filling of dentinal tubules. G6, G9, and G12 appeared to undergo demineralization and exposure

of dentinal tubules. Lastly, G10 and G11 showed an increase in inorganic ions, with G10 showing the most expressive increase in matrix inorganic ions. These results were very similar to those of G1/Control, apart from an abrupt decrease in the percentage of the organic ion carbon (Figure 2).

The ions' local environment affected each group's resonance frequency fingerprint, which became sensitive to compositional and structural alterations induced by the protocols. Compared to the Raman mode spectra from G1/Control, the most evident alterations were detected in G3, G10, G11, and G12. The degradation of dentin quality was evident in G3, reducing the efficiency of Raman emission from the sample surface, leading to noisier spectra, and affecting mode profiles. The 10–20% increase in intensity ratios of modes associated with collagen organic groups suggests a substantial decrease in the quantity and quality of the mineral dentin component. G10's procedure caused the most positive alterations in the dentin (Figure 3-A).

Comparing the XRD spectra obtained under the same acquisition parameters, G1/Control, G4, G6, and G10 showed sharpening, deconvolution, and discretization of diffraction peaks. On the other hand, G2, G3, G5, and G12 suggested some contraction of the cell lattice volume and possibly degradation of crystallization quality (Figure 3-B).

Discussion

Biomimetic remineralization has emerged as a promising strategy to reverse the adverse effects of caries lesions by restoring the structure and mechanical properties of demineralized dentin.[13] Our pilot study aimed to assess morphological and structural changes in dentin following two chemical ACAD protocols, evaluate the effects of three BRPs—CPP-ACP, SAP-P_{11}-4, and Ca/P-PILP—on dentin structure, and determine the most favorable trend of the ACAD-BRP combination.

While XRD diffractograms are mainly correlated to the long-range mineral lattice order, MSR spectroscopy can be sensitive to dentin's inorganic and organic constituents. MRS studies the assembly of allowed vibration modes characteristic of a molecule/structural frame that defines an active Raman oscillator. Generally, a relative change occurring in such structures will translate into the respective Raman modes, varying in peak center (xc) and width (FWHW) compared to the reference mode. A major narrowing of peaks corresponds to a decrease in structural disorder, while an increase in mode wavenumber can correspond to a rigid oscillator related to stronger or less flexible molecular bonds.[14,15]

The sharpening, deconvolution, and discretization of diffraction peaks in XRD diffractograms correlated by Scherrer[16]

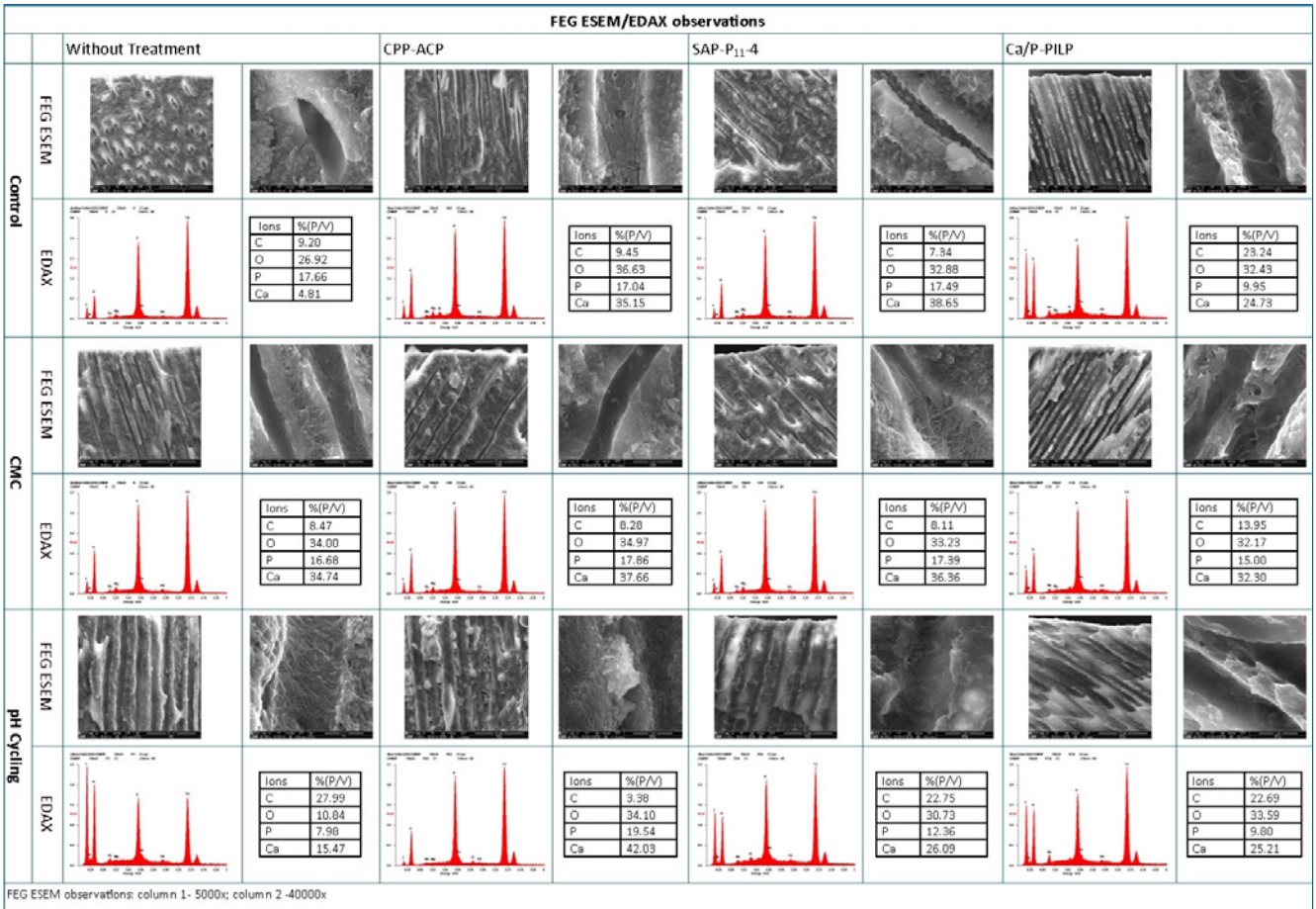

Figure 2. FEG ESEM/EDAX observations

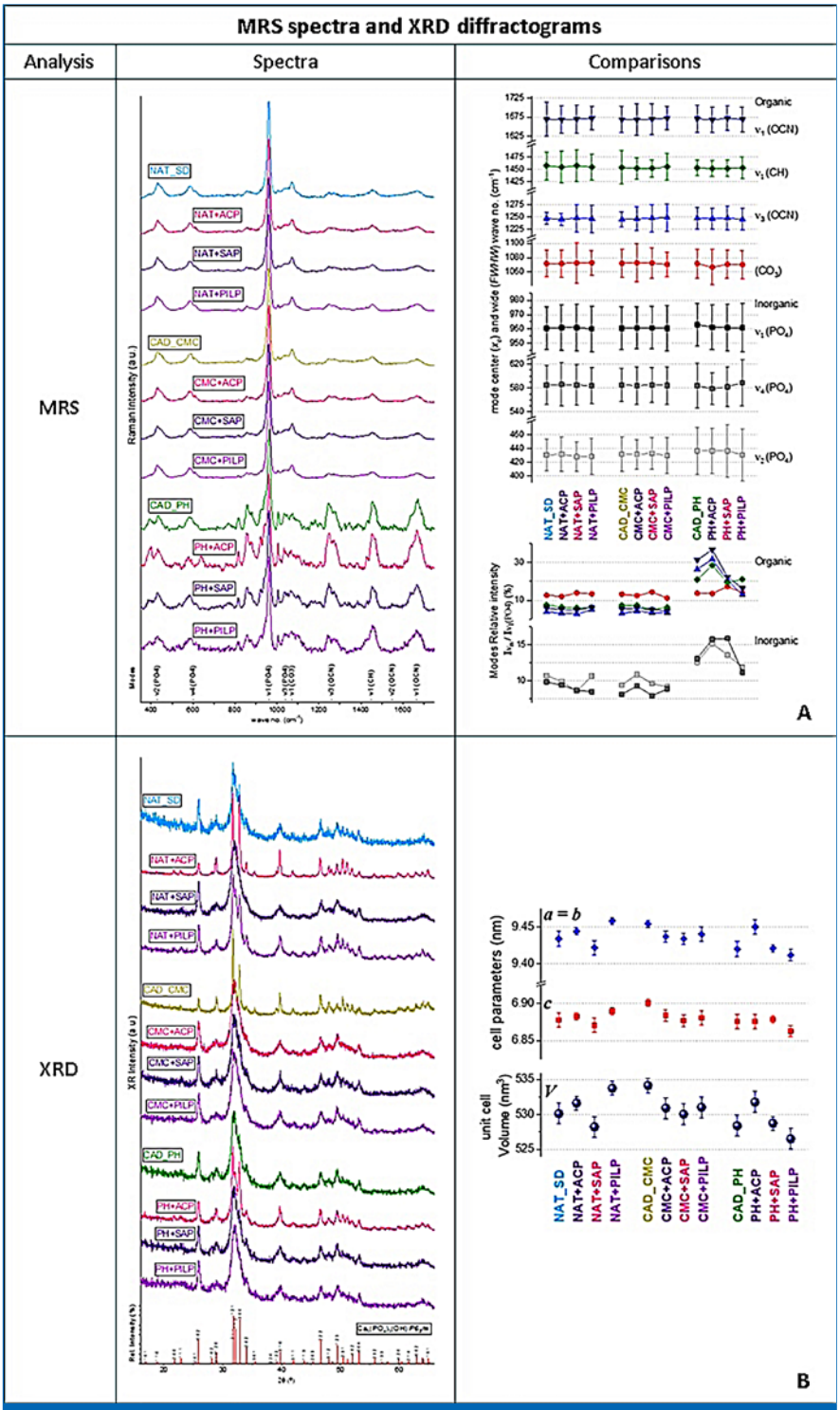

Figure 3. MRS spectra and XRD diffractograms. «A. Comparison of representative Roman module parameters in the spectral region between 300 and 1750 cm^{-1}. A simplified interpretation was based on the intensity/dimension of some modules' peaks as an indication of the presence/absence of the oscillator that originates the module[34,35], i.e., phosphate $(PO_4)^{3-}$ (u2 at 400–470 cm^4, u4 at 520–600 cm^{-1}, and u1 and u3 at 960-1100 cm^{-1}), and modes related to the organic radicals: carbonate $(CO3)^2$ at ~1070 cm^{-1}, carboxyl (CH) at ~1450 cm^{-1}, and amide (OCN) at 1240–1270 cm^{-1}, 1500–1550 cm^{-1}, and 1600–1700 cm^{-1}. B. Structural parameters of the hydroxyapatite component obtained by a detailed Rietveld analysis.[15] The indexation was made to the polycrystalline phase of the hexagonal space group P_{63}/m (176) and composed of two formula units, with formula $Ca_5(PO_4)^3(OH)$, according to reference.[36,37]»

result from a notable growth in crystallite average size, decreasing the contribution to lattice distortions of grain boundaries. These XRD Rietveld analyses reveal important recrystallization with a lattice volume expansion of +0.3 to +0.8% and an improvement in the quality of hydroxyapatite crystallization. Generally, these losses of inorganic ions, or degradation of hydrogen bridges between molecules, promote repulsion in the framework of anionic oxygen (a practical indication of dentin microstructure regeneration).

The results of our study reject the null hypothesis, demonstrating clear morphological and structural differences compared to natural dentin. Both chemical ACAD protocols increased surface demineralization and altered dentin morphology and structure. The literature suggests that pH cycling is more effective than acidified gel because it produces a thicker layer of demineralization. Our findings agree that pH cycling might be more appropriate than other procedures to simulate a substrate after a caries lesion.[17] This chemical model effectively reproduced the dynamic processes involved in caries progression, closely mimicking in-vivo demineralization conditions.[18]

While both Ca/P-PILP and SAP-P_{11}-4 demonstrated efficacy in altering dentin mineral composition, they did not achieve the same level of efficacy as CPP-ACP in promoting remineralization. According to the literature, P_{11}-4 can interact with type-I collagen, boosting the hybrid layer's stability and the fibers' resilience to proteolysis while supporting mineral development.[19] The functional domain of natural proteins controlling the initiation and progression of the biomimetic remineralization process in Ca/P-PILP can be replicated by non-collagenous-protein analogs with high affinities for Ca^{2+} and collagen.[18,20] These analogs also act as inhibitors of ACP nanoparticle aggregation.[21]

Furthermore, combining CPP-ACP with pH cycling showed superior remineralization effects, agreeing with previous literature.[9,22-24] CPP-ACP exhibits synergistic effects in promoting dentin remineralization, as the acidic environment during demineralization facilitates the release of calcium and phosphate ions from CPP-ACP.[25,26] These ions can then be deposited onto the demineralized tooth surface during remineralization, promoting hydroxyapatite crystal formation.[24,27] Studies have consistently demonstrated that the combination of CPP-ACP and pH cycling leads to increased mineral uptake and improved remineralization of dentin lesions compared to either treatment alone. Additionally, CPP-ACP has been found to inhibit cariogenic bacteria's growth and reduce caries lesions' progression, emphasizing its value as an adjunct in managing caries-affected dentin.[22,24]

Our results suggest that CPP-ACP may offer a clinically viable approach to managing caries-affected dentin. By effectively reversing the demineralization process and promoting remineralization, CPP-ACP has the potential to arrest caries progression, thereby preserving dentin structure and function.[25]

This study aimed to assess the trend toward a favorable ACAD-BRP combination. It should be noted that this is a preliminary study with a consequently small sample size. Therefore, the limitations of this pilot in-vitro study should be acknowledged, and the interpretation of the results should be cautious. Further studies with larger sample sizes are warranted to validate our findings.[28-37] Additionally, future research should consider varying the experimental conditions, such as different pH levels, times or temperatures, to better understand the full potential and limitations of these biomimetic remineralization protocols in diverse clinical scenarios.

Nevertheless, our study highlights the potential of CPP-ACP as a valuable adjuvant in caries lesion remineralization and the importance of considering dynamic environmental factors, such as pH fluctuations, in experimental models evaluating BRP.

Conclusions

The results show changes in dentin morphology and structure, confirming the potential effectiveness of the two ACAD chemical protocols in inducing changes in dentin, with the pH cycling protocol being more promising. Different degrees of efficacy were found between the BRPs, with CPP-ACP emerging as the most promising agent for promoting remineralization and restoring dentin structure in both ACADs. The combination of pH cycles with CPP-ACP showed a superior ability to promote dentin remineralization compared to the other combinations evaluated in this in-vitro study.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

CRediT authorship contribution statement

Rosário Costa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Sofia**

Arantes-Oliveira: Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **Lisete Fernandes:** Data curation, Formal analysis. **Fábio G. Figueiras:** Data curation, Formal analysis, Writing – original draft. **João Cardoso Ferreira:** Conceptualization, Validation, Supervision, Writing – review & editing. **Paulo Ribeiro de Melo:** Conceptualization, Methodology, Validation, Supervision, Project administration, Writing – review & editing.

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