Results in Chemistry 3 (2021) 100232

Contents lists available at ScienceDirect

Results in Chemistry

journal homepage: www.sciencedirect.com/journal/results-in-chemistry

In vitro polarized Raman analysis for the evaluation of the efficacy of CPP-ACP remineralizing mousse in tooth hypomineralization

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ARTICLE INFO

Keywords:
Raman spectroscopy
Molar incisor hypomineralization
CPP-ACP tooth mousse
Remineralization

ABSTRACT

The purpose of this *in vitro* study is to evaluate the efficacy of a commercially available tooth mousse in the remineralization of human enamel in cases of Molar-incisor-hypomineralization (MIH).

Treatment protocol with GC Tooth Mousse containing Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) was applied to 12 teeth (4 healthy, 4 hypomineralized with white opacities and 4 hypomineralized with yellow opacities) and the samples were evaluated with polarized Raman microscopy. The depolarization ratio of the symmetric stretching band of phosphate band was compared in each sample before and after treatment with the tooth mousse.

The mean depolarization ratio values decreased in all three groups, after the treatment protocol, with significant differences ($p < 0.05$) for the two hypomineralized teeth' groups. These results allowed us to conclude that there was an improvement in mineral density and organization of the hypomineralized enamel after treatment with CPP-ACP tooth mousse.

Introduction

Molar incisor hypomineralization (MIH) is defined as the hypomineralization of one to four permanent first molars frequently associated with affected incisors [1]. This hypomineralization occurs when systemic factors affect the ameloblastic activity during the maturation stage of amelogenesis [2]. This results in an increase in enamel porosity, turning those teeth more vulnerable to abrasion and carious lesions. Due to the increased porosity of the hypomineralized enamel there is also a chronic inflammation of pulpal tissues [3] making these teeth extremely sensitive: arduous oral hygiene leading to accumulation of dental plaque and difficult to anesthetize [1,3]. In more severe cases, the porous enamel may even fracture, creating post-eruptive enamel breakdowns [1].(1)

The prevention, early diagnosis and management of this condition is crucial to keep these teeth in the best possible conditions and without associated pain [1,4]. Current MIH diagnosis, in a clinical setting, includes the observation of opacities ranging from white to brownish shades, due to an increase of the organic structure on the enamel layer: sound enamel in composed of 80–90% of carbonated hydroxyapatite, whilst in these teeth the levels of serum albumin, alpha 1 antitrypsin and type 1 collagen are increased, and in teeth with yellow or brown opacities, antithrombin 3 protein has also been detected [2].

Regarding opacity management and treatment, casein phosphopeptide amorphous calcium phosphate (CPP-ACP) has been essayed for the remineralization to the most superficial surface of the enamel, acting as a depot of calcium and phosphate, and stabilizing high concentrations of these ions [5–9].

The literature suggests that the application of CPP-ACP pastes decreases the loss of mineral, the depth, and width of lesions [10]. It has even been showed that with the consistent use of CPP-ACP there is an increase of mineral density and demineralization inhibition [11,12].

Kutuk et al. in 2018 observed that treating enamel with fluoride or CCP-ACP prevented the decreased of microhardness after bleaching [9] and in the same year, Yu et al. showed that the application of CPP-ACP reduced surface softening and morphologic changes of the teeth exposed to erosion [13].

Raman spectroscopy has been used to study the changes in the

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https://doi.org/10.1016/j.rechem.2021.100232
Received 30 September 2021; Accepted 1 November 2021
Available online 6 November 2021

symmetric stretching mode of the tetrahedral phosphate group (PO_4^{3-}) which is representative of the mineral phase in teeth. Several studies evaluated these modifications with real [14] or artificial [15] carious lesions and external demineralizing agents, such as acidic beverages [16], bleaching agents [17] and gustatory stimulants of salivary secretion [18].

More significantly, the analysis of the PO_4^{3-} symmetric stretching band at 959 cm^{-1} and its polarization anisotropy have also been shown to be sensitive markers of demineralization, namely of early caries lesions [14,19]. Studies have shown an increase of the depolarization ratio of this band in demineralized enamel, prompting the use of this methodology for caries diagnosis.

The use of Raman spectroscopy has already been attempted to evaluate the efficacy of CPP-ACP based treatments. Job et al. in 2018 observed that CPP-ACP increased the phosphate content of demineralized teeth, through the intensity of the peak of the Raman band after the treatment, deducing there was hydroxyapatite formation [20]. However, when comparing peak intensities, instrumental variations and sampling heterogeneity might compromise the evaluation of the spectra. It is known that different teeth or even different locations on one tooth can have different Raman scattering efficiency due to bio- heterogeneity, therefore the measured Raman intensity could vary from person to person or even from tooth to tooth. In contrast, comparing peak ratios avoid these variations as the depolarization ratios respond solely to the underlying structure of enamel.

To the best of our knowledge, Raman microscopy was not yet employed in the evaluation of different MIH opacities or remineralization protocols.

The objective of the present study is to understand, through Raman spectroscopy analysis, if our protocol of application of CPP-ACP, is effective in remineralizing hypomineralized enamel in two types of MIH lesions.

Materials and methods

Specimen selection

The study sample consisted of 12 M teeth that were selected and allocated in one of the three groups: Group A – 4 healthy teeth - extracted for orthodontic reasons; Group B – 4 teeth with MIH presenting white hypomineralized opacities; Group C – 4 teeth with HIM presenting yellow hypomineralized opacities

Saliva collection

A saliva collection protocol was carried out. The donor had to floss and brush his teeth with a soft brush, a fluoridated toothpaste and wait for an hour without eating or drinking. Saliva was collected 5 min after the donor swallowed all the saliva in the oral cavity. This protocol was made as many times as necessary to obtain enough quantity of saliva for this study.

Prophylactic protocol

Previous to the Raman analysis, teeth went through a prophylactic protocol. A polishing brush with pumice paste in low rotation handpiece (0,04 mg of pumice; 0,8 mL of water) was used for 60 s. Samples were then washed with distilled water for 60 s and submitted to an ultrasound bath in ethanol 100% for another 60 s using the Bransonic® M2800-E, Emerson, electronic cleaning device. Samples were stored in Hanks' solution in the refrigerator for up to 3 days until Raman measurements. Buchwald et al [21] evaluated the influence of storage solution on human enamel, considering the several parameters that are evaluated with Raman microscopy and found no significant differences after storage in Hank's solution for a period up to 90 days.

CPP-ACP treatment protocol

The sample was placed in a Petri dish where enough saliva was added to cover all of the sample's surface, and a pea size of GC Tooth Mousse containing CPP-ACP (GC, Tokyo, Japan) was applied, with a swab, so that a 1 mm even layer covered all the surface of each sample for 3 min. After this, the necessary amount of saliva was added, to cover all sample's surface. The saliva and Tooth Mousse were mixed for 10 s with a swab, followed by a 30-minute rest. Each sample was then washed for 15 s with distilled water, dried with absorbent paper, placed in a holder, and analyzed again with the Raman microscope to determine the depolarization ratio after treatment.

Confocal Raman microscope

Raman spectra of samples were obtained from a Horiba XploRA Confocal Microscope using the near infrared laser (785 nm) with a 1200 lines/mm grating. This way, the spectral range investigated was from 200 cm^{-1} to 1300 cm^{-1} with spectral resolution of 4 cm^{-1}. Using an entrance slit of 100 μm, and a confocal hole of 300 μm, the scattered light collected by the objective was dispersed onto the air-cooled CCD array of an Andor iDus detector. A 100x objective (N.A. = 0.9) was used to focus on the surface of enamel, as well as a 50% neutral density filter rendering an incident power on the sample of 5.0 ± 0.4 mW (laser-check®, Edmund optics). Each spectrum was obtained by 3 accumulations of 20 s each and 20 spots were inspected for each sample.

Each sample was removed from the Hanks' solution, washed with distilled water for 5 s, dried with absorbent paper, placed on holder, and analyzed with Raman microscopy to determine the depolarization ratio before treatment and afterwards following treatment with CPP-ACP.

In order to determine the depolarization ratio (ρ) of the most intense band in the Raman spectrum, assigned to the symmetric stretching band of phosphate ions ($\nu_1 \sim 959$ cm^{-1}), in each spot, spectra were recorded for two orthogonal polarizations of scattered light (perpendicular and parallel to the polarization of the incident laser). The ρ959 was then determined according to [22]:

$$\rho 959 = \frac{I_{959}\perp}{I_{959}\mathrm{II}}$$

where I_{959} II is the intensity of the Raman band at ~959 cm^{-1} using parallel polarization and $I_{959}\perp$ is the intensity of the Raman band at ~959 cm^{-1} using perpendicular polarization between the incident laser and the scattered radiation.

Statistical analysis

Statistical analysis was performed with SPSS v26.0. First, a Shapiro-Wilk test for normal distribution evaluation was performed. Considering all the variables tested, the values obtained for the healthy teeth, white opacities and post-treatment yellow opacities presented normal distribution while the pre-treatment yellow stains results could not be assumed as normally distributed. Paired Student *t*-test was performed for the evaluation of the healthy teeth and white opacities, before and after the treatment protocol, and Wilcoxon Sign rank test was used for the yellow opacities' results. A significance level of 0.05 was considered.

Results

The spectra recorded for a sample presenting a hypomineralized yellow opacity before (Fig. 1) and after (Fig. 2) treatment with GC Tooth Mousse containing CPP-ACP is showed, after baseline correction. Table 1 presents the band assignments for the spectra [17]. The Raman band of interest corresponding to the symmetric stretching of phosphate is highlighted (ν_1 PO_4^{3-} ~ 959 cm^{-1}) while other vibrational modes of phosphate and carbonate are identified but were not considered in the

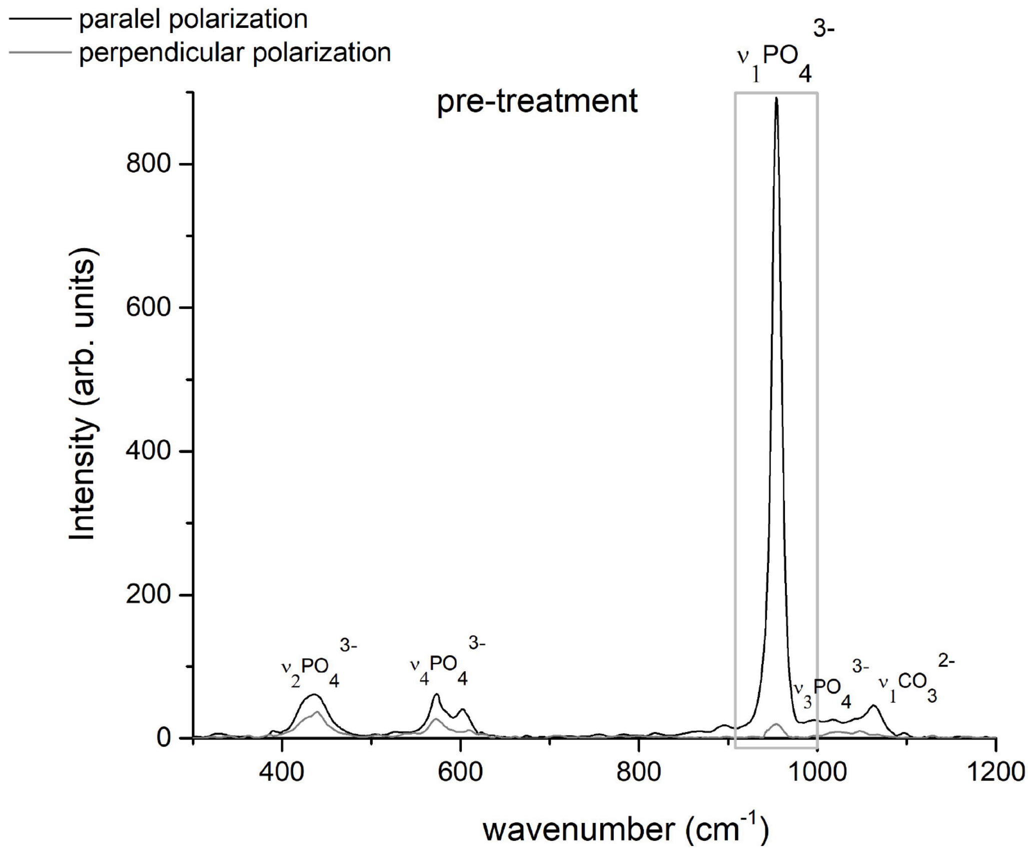

Fig. 1. Comparison of polarized Raman spectra of enamel presenting yellow opacities before treatment with CPP-ACP.

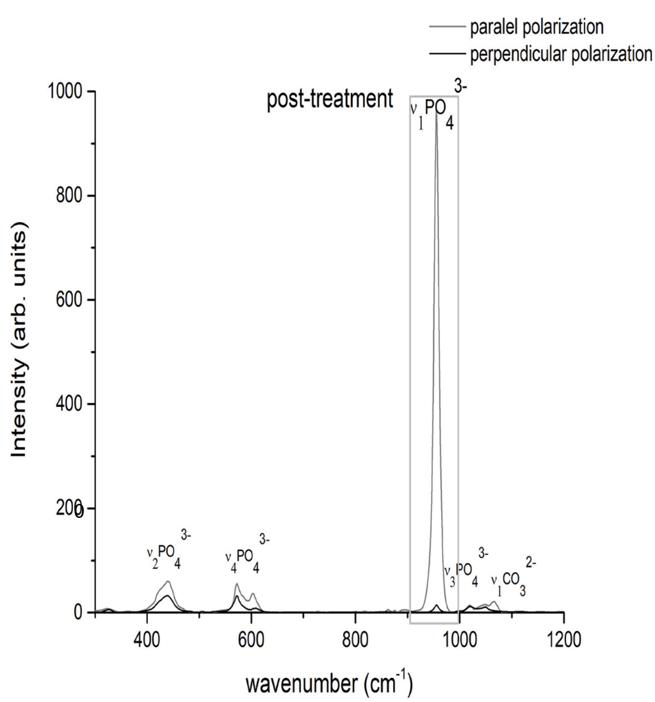

Fig. 2. Comparison of polarized Raman spectra of enamel presenting yellow opacities after treatment with CPP-ACP.

evaluation of the enamel due to their lesser sensitivity [19]. As can be seen, there is an increase of the main band in parallel mode concomitant with a decrease of the same band in perpendicular mode, after

Table 1
Assignment of the characteristic bands of Hydroxyapatite for the spectra in Figs. 1 and 2.

Band (cm^{-1})	Assignment
431, 449	doubly degenerate bending mode (ν_2) of the PO_4^{3-} group (O-P-O) bond
582, 595, 612	triply degenerate bending mode (ν_4) of the PO_4^{3-} group (O-P-O) bond
959	symmetric stretching mode (ν_1) of the PO_4^{3-} group P-O bond
1027, 1047, 1075	triply degenerate asymmetric stretching mode (ν_3) of the PO_4^{3-} group P-O bond
1072	symmetric stretching mode (ν_1) of the CO_3^{2-} group

treatment.

The results for the mean depolarization ratio of the symmetric stretching band of phosphate for the three studied groups are presented in Table 2.

Table 2
Mean depolarization ratio of the symmetric stretching band of phosphate values for the three groups. Uncertainty is given by the standard deviation of the distribution (SD).

Study Group	Depolarization ratio Pre-treatment (mean values ± SD)	Depolarization ratio Post-treatment (mean values ± SD)	Significance level (p)
A) Healthy teeth	0.020 ± 0.004	0.019 ± 0.003	0.06
B) White opacity teeth	0.023 ± 0.003	0.018 ± 0.003	<0.001
C) Yellow opacity teeth	0.03 ± 0.01	0.026 ± 0.006	<0.001

The mean value of the depolarization ratio for the healthy teeth group before treatment was 0.020 and after treatment 0.019, with a standard deviation of 0.004 and 0.003, respectively. There was no statistically significant difference ($p > 0.05$) in the enamel mineralization of the healthy teeth group before and after treatment.

For group B, the hypomineralized teeth with white opacities, the mean values obtained for the depolarization ratio before and after treatment were, respectively, 0.023 and 0.018, with a standard deviation of 0.003 in both situations. In this case there was a significant decrease ($p < 0.05$) in the mean values of depolarization ratio, which suggests an improvement in the enamel mineralization after treatment.

Also, in group C, with teeth presenting yellow hypomineralized opacities, there was a significant decrease ($p < 0.05$) in the mean values of depolarization ratio after treatment, with values ranging from 0.03 before and 0.026 after treatment, with a standard deviation of 0.01 and 0.006, respectively. Also, in this case there was an improvement in the enamel mineralization with treatment.

Discussion

Using Raman analysis, we were able to observe that the treatment protocol used for CPP-ACP application had a positive and significant effect in reducing the depolarization ratio on enamel surfaces with either white or yellow hypomineralized opacities.

In sound enamel, apatite crystals are methodically organized perpendicular to the enamel surface revealing a high anisotropy of the symmetric stretching band of phosphate. It has been shown, using Raman spectroscopy, that the arrangement of apatite crystals is altered in demineralized enamel, since the structure is more disordered, and the crystals are not parallel to one another. Therefore, in demineralized enamel, the value of the depolarization ratio is higher [16,18,19].

The molar-incisor hypomineralization affected teeth, with opacities, have in their microstructure unorganized enamel rods of hydroxyapatite crystals, that lead to a reduction in mechanical properties like hardness and modules of elasticity [1]. Likewise, according to Cvek *et al.* caries resistant teeth have a larger proportion of the microcrystals well oriented, meaning that the degree of hydroxyapatite microcrystals alignment is a property that may help determine the tooth mechanical resistance [23].

By comparing the depolarization ratio values, it was possible, to observe an increase of organization of the enamel apatite crystals after CPP-ACP treatment, a parameter that has been successfully used to diagnose early caries lesions in teeth [14,15] and has now proven suitable for the evaluation of the remineralizing effect of CPP-ACP.

CPP-ACP has the capacity to adhere to the enamel and supersaturate the environment with free calcium and phosphate ions, which suppresses the process of demineralization and promotes remineralization. The presence of CPP-ACP allows a fast return of calcium resting concentrations and early remineralization [7,24].

The obtained results of this study are indicative that the hypomineralized opacities mechanical resistance increased after CPP-ACP treatment.

Moreover, it was possible to decrease the white opacities depolarization ratio mean values down to the level of the healthy teeth mean values, and the yellow opacities mean values to the level of the white opacities values, before treatment.

In future studies, the used protocol should evolve, by increasing the time of application, or the number of applications to try to improve the yellow opacities depolarization ratio mean values and achieve values as low as the healthy teeth depolarization ratio mean values.

The used protocol was based on the fabricant instructions, and with it, was possible to decrease the samples depolarization ratio, which means improving the alterations in the degree of crystallite orientation. A similar protocol was used by Yu *et al.* in 2018, concluding that the application of the protocol with CPP-ACP had reduced the surface softening and morphology changes of the human enamel after erosion [13]. In other study, Vieira *et al.* in 2017, tested many protocols, being one of them similar to this one, and the author observed that this protocol had better results in surface hardness after pH-cycling, percentage of surface hardness recovery and integrated loss of subsurface mineral than the placebo group and the groups where CPP-ACP was applied for 3 and 8 h [24].

In this *in vitro* study, human saliva was used instead of artificial saliva formulations. Batista *et al.* in 2016 observed that storage of samples in human saliva resulted in significantly less mineral loss and that artificial saliva formulations are generally unable to form the pellicle that acts as a diffusion barrier, inhibiting the contact of acids with the dental surface [25].

Conclusions

Based on this *in vitro* study and the evaluation of the polarized Raman results, we can confirm that the determination of the depolarization ratio of enamel is a sensitive tool for the evaluation of the soundness of enamel – there was an increase of this parameter in the samples presenting molar-incisor-hypomineralization (MIH). More remarkable is the improvement of the degree of crystallite orientation in both white and yellow enamel opacities after treatment with CPP-ACP tooth mousse. This is the reverse behavior evidenced profusely in literature after enamel demineralization, leading to the conclusion that there was a significant remineralization of enamel after only one application of CPP-ACP tooth mousse.

CRediT authorship contribution statement

Sara Franco: Formal analysis, Data curation. **Inês C. Martins:** Methodology. **Sofia Arantes-Oliveira:** Conceptualization. **Sofia Pessanha:** Formal analysis. **Paula F. Marques:** Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia (FCT) (Portugal) through the research centre grant UID/FIS/ 04559/ 2020 to LIBPhys from the FCT/MCTES/PIDDAC, Portugal. I. Cardoso-Martins acknowledges Cuf, SA for the PhD scholarship.

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