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Research Article

Non-Invasive Treatment of Reversible Caries Lesions *in Vitro*: A Novel Era in Denal Practice

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Abstract

Background: The efficacy of GC Tooth Mousse cream (CPP-ACP) as a remineralizing agent has been affirmed. Recently, nano-hydroxyapatite-containing dentifrice "KAREX" has been put on the market as a dental care product suitable for dental tissue renovation. ***Objective***: Using an in vitro caries model to compare the remineralizing effect of the two products. ***Methods***: 12 sound premolars were exposed to pH cycling model to induce subsurface lesions. Thereafter, teeth were randomly divided into 2 groups scanned by Raman microscopy two times: once after initial caries induction and once again after intervention to provide phosphate maps showing the net differences between affected and unaffected enamel areas. Each specimen was treated with the respective remineralizing agent for 5 min every 24 h for 21 days. The prominent phosphate peak at 960 cm^{-1} was nominated to observe changes in its intensity. ***Results***: The width of the phosphate peak measured by FWHM was calculated across each spectral map to evaluate the effect of remineralizing agents on the crystalline structure of demineralized enamel. At the end of the treatment, a significant difference has been attained in respect to phosphate gain in the body of lesions treated by nHA-containing dentifrice compared to the counterpart treated by CPP-ACP. However, no significant differences were observed among the treatment groups with regard to enamel crystallinity. ***Conclusions***: Enamel surface layer permeability along with material consistency might represent key factors in subsurface lesion remineralization.

Keywords: CPP-ACP, Confocal Raman microscopy, Full-width at half-maximum, Non-invasive treatment, nHA containing-dentifrice, Phosphate maps.

العلاج غير الجراحي لحالات التسوس القابلة للعكس في المختبر: حقبة جديدة في ممارسة طب الأسنان

الخلاصة

الخلفية: تم تأكيد فعالية كريم موس الأسنان GC (CPP-ACP) كعامل إعادة تمعدن. في الآونة الأخيرة، تم طرح معاجين الأسنان المحتوية على النانو هيدروكسيباتيت "KAREX" في السوق كمنتج للعناية بالأسنان مناسب لتجديد أنسجة الأسنان. **الهدف**: استخدام نموذج التسوس في المختبر لمقارنة تأثير إعادة التمعدن للمنتجين. **الطرائق**: تم تعريض 12 من الضواحك لنموذج دورة الأس الهيدروجيني للحث على آفات تحت السطحية. بعد ذلك، تم تقسيم الأسنان بشكل عشوائي إلى مجموعتين تم مسحهما ضوئيا بواسطة مجهر رامان مرتين: مرة بعد تحريض التسوس الأولي ومرة أخرى بعد التدخل لتوفير الفوسفات التي توضح الاختلافات الصافية بين مناطق المينا المصابة وغير المصابة. تمت معالجة كل عينة بعامل إعادة التمعدن المعني لمدة 5 دقائق كل 24 ساعة لمدة 21 يوما. تم ترشيح ذروة الفوسفات البارزة عند 960 سم $^{-1}$ لمراقبة التغيرات في شدتها. **النتائج**: تم حساب عرض ذروة الفوسفات المقاسة بواسطة FWHM عبر كل خريطة طيفية لتقييم تأثير عوامل إعادة التمعدن على التركيب البلوري للمينا منزوعة المعادن. في نهاية العلاج، تم تحقيق فرق كبير فيما يتعلق بكسب الفوسفات في جسم النخر المعالجة بالأسنان المحتوية على nHA مقارنة بالنظير الذي يعالج CPP-ACP. ومع ذلك ، لم تلاحظ فروق ذات دلالة إحصائية بين مجموعات العلاج فيما يتعلق بتبلور المينا. **الاستنتاجات**: قد تمثل نفاذية الطبقة السطحية للمينا جنبا إلى جنب مع تناسق المواد عوامل رئيسية في إعادة تمعدن التسوسات تحت السطحية.

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INTRODUCTION

White spot lesion (WSL) represents an early stage of caries formation where an intact surface layer (ISL) overlays subsurface enamel demineralization, which takes place due to an imbalance between pathological and protective factors [1]. Non-invasive treatment of WSL through remineralization represents the aim of modern dentistry. The clinical usage of calcium phosphate salts in the enamel remineralization process was unsuccessful because these salts are insoluble and unable to provide bioavailable ions, which are essential

for enamel restoration. Novel technology assists in localizing bioavailable ions at the tooth surface to promote enamel renovation, which becomes mandatory [2]. Dairy products are among the nutrients most recognized as exhibiting anticariogenic activity [3]. The components largely responsible for this activity have been identified as bovine milk phosphoprotein, casein, calcium, and phosphate (3-5). Casein phosphopeptides (CPP) containing the cluster sequence – Ser(P)-Ser(P)- Ser(P)-Glu-Glu – have a remarkable ability to stabilize amorphous calcium phosphate (ACP) in a metastable solution. The CPP binds high amounts of calcium and phosphate to form nanoclusters of ACP through the multiple phosphoseryl residues, preventing their growth to the critical size required for nucleation and precipitation [3]. Thus, CPP tends to increase calcium and phosphate levels in the subsurface lesions, thereby esthetically repairing WSLs [6,7]. This tasteless, stable, and highly soluble CPP-ACP has been registered as Recaldent® and commercialized in dental professional products (Tooth Mousse®). GC Tooth Mousse cream containing 10% w/w CPP-ACP has been used clinically to treat mild to moderate fluorotic tooth enamel without surgical intervention [2]. Nano-hydroxyapatite (nHA) is one of the most biocompatible and bioactive materials that have a similarity to the apatite crystal of tooth enamel in morphology, crystal structure, and crystallinity [8]. Recently, many reports have shown that nHA has the potential to remineralize artificial carious lesions following addition to toothpastes and mouthwashes [9,10]. nHA displays high affinity to the tooth and can strongly adsorb on enamel surfaces [11], promoting remineralization due to their size-specific effects corresponding to enamel ultrastructure [12]. Toothpaste comprising nHA has been commercially available in Japan since the 1980s, and was approved as an opposing caries agent in 1993 based on randomized anti-caries field trials in Japanese schoolchildren [13]. KAREK is a caries-inhibitory nHA-containing dentifrice, lately produced by Zahnpasta (dental care products, Bielefeld, Germany). It has antimicrobial effects thanks to the presence of active substances zinc and xylitol. This hydroxyapatite (HA)-based toothpaste forms a protective layer on tooth surfaces and can initiate regeneration of attacked areas even in case of low salivary flow rates because the active ingredient is already present in mineral form and does not have to be formed from the saliva through remineralization processes [14]. Confocal Raman microscopy is a powerful technique that measures the inelastic scattering of incident light, which is used to analyze the internal structure of mineralized tissues. The spatial resolution of 300 nm makes it a novel optical approach appropriate for incipient caries detection [15]. The efficacy of Tooth Mousse® cream as a potent remineralizing agent has been affirmed by many regulatory bodies around the world. Therefore, the purpose of the current study was to determine whether newly produced KAREX toothpaste can remineralize artificial subsurface enamel lesions and compare its remineralizing effect with GC Tooth Mousse cream, being used as a criterion in this study.

METHODS

Treatment procedure

12 sound premolars were extracted for orthodontic treatment and collected with patients' informed consent and approval of the local ethical research committee (Comité de protection des personnes-CPP Sud Mediterranee IV, process No. 2017-2907) before usage. All procedures were carried out in accordance with relevant guidelines and regulations. Teeth were cleaned of debris and stored in deionized water with 0.1% antimicrobial thymol at 4°C until use. Teeth were exposed to a pH cycling chemical model to induce uniform, reproducible subsurface enamel lesions *in vitro* using sequential series of exposure to de/remineralizing solutions. The regimen was repeated for a period of 6 days, the temperature maintained at 37°C [15]. Thereafter, teeth were randomly divided into 2 groups and cut into two, each part being scanned by Raman microscopy two times: once after initial caries induction and once again after intervention to decrease sources of variation. GC Tooth Mousse cream (Recaldent®, CPP-ACP) and KAREX toothpaste (dental care products, Griesheim, Germany) were applied to remineralize the formerly created lesions. Each specimen was treated with the respective remineralizing agent for 5 min every 24 h for 21 days, with the help of a cotton applicator tip. Treated samples were washed with distilled water and placed in artificial saliva at 37°C. Artificial saliva was renewed every 24h just before immersion of freshly treated samples [16]. The artificial saliva was prepared according to a method given by Sato [17], using inorganic components similar to that of natural saliva. 3.90 mM Na_3PO_4, 4.29 mM NaCl, 17.98 mM KCl, 1.1 mM $CaCl_2$, 0.08 mM $MgCl_2$, 0.05 mM H_2SO_4, 3.27 mM $NaHCO_3$, pH = 7.2. All chemical products were supplied by Sigma-Aldrich, France.

Raman microscopy

Raman spectra were recorded using a Witec Confocal Raman Microscope System (Witec, Ulm, Germany). Excitation was assured by a frequency-doubled Nd:YAG visible laser (Newport, Evry, France) at 532 nm. Acquisition time of a single spectrum was adjusted to 0.05 s. Chemical mapping of dental enamel was carried out over the enamel cross-section from the outer surface toward the DEJ. The prominent phosphate peak at 960 cm^{-1} attributed to symmetric stretching mode 1, was nominated as the inner standard to observe changes in PO_4^{3-} intensity. Furthermore, the width of the same PO_4^{3-} peak was measured across each

spectral map to evaluate the effect of remineralizing agents on the crystalline structure of demineralized enamel. As peak width increases, crystallinity decreases [18]. An indicative standardization bar showing the maximum and minimum sums of phosphate intensity across the enamel film was adopted. Representative Raman spectra were collected from the damaged and healthy enamel sites and enhanced after baseline correction using SpectraGryph optical spectroscopy software, Version 1.2.7, 2017. Thereafter, phosphate intensity at 960 cm^{-1} along with ($FWHM^{-1}$) values were obtained twice, once after artificial caries induction and over again after treatment with the remineralizing agents to detect the net differences in enamel inorganic component.

Viscosity measurement

Viscosity of remineralizing agents was determined using FUNGILAB viscometer (FUNGILAB S.A. Ind., Spain). Spindle number (R7) was used and the rotation speed of the spindle was adjusted to 6.0 RPM.

Statistical analysis

Statistical analysis was performed using the One-Way ANOVA test for normally distributed data and the Kruskal-Wallis ANOVA for data that were not normally distributed. All statistical procedures were performed at an overall significant level of $\alpha = 0.05$ with SigmaPlot version 11.0 (Systat Software, Inc., USA). The difference (D) between the initial PO_4^{3-} intensity (I_i, after pH cycling) and the final intensity (I_f, after treatment with the selected agents) was calculated for each specimen, and the mean of this difference ($\bar{D}$) was calculated for each of the experimental groups. The ratio of the difference ($\bar{D}$) to the ($\bar{I_i}$, mean of Ii for each experimental group) was also calculated for each experimental group, which represents the rate of change in phosphate intensity of the specimens [19].

$$(D) = I_f - I_i \ldots\ldots\ldots (1)$$

$$(\bar{D}) = \sum (D)/n \ldots\ldots\ldots (2)$$

$$(\bar{I_i}) = \sum (I_i)/n \ldots\ldots\ldots (3)$$

$$\% \text{ rate of change in the intensity (CI)} = (\bar{D})/(\bar{I_i})*100 \ldots (4)$$

The same calculation was repeated to measure the rate of change in FWHM value, which is inversely proportional to enamel crystallinity, as elucidated in Table 1.

Table 1: **T**he rate of change in phosphate intensity & full width at half-maximum (FWHM) values after treatment with different remineralizing agents estimated by special equation

Remineralizing agent plus affected zone	Rate of change in PO_4^{3-} intensity (%)	Rate of change in FWHM (%)
CPP-ACP (ISL)	+9.20	-2.60
nHA based dentifrice (ISL)	+39.70	-0.50
CPP-ACP (Body of lesion)	-8.10	-2.30
nHA based dentifrice (Body of lesion)	+96.60	-0.30

ISL: Intact Surface Layer.

RESULTS

Phosphate maps were constructed from phosphate peak intensity at 960 cm^{-1} before and after remineralization (Figure 1). Phosphate intensity reveals its amount within the inorganic crystals and is directly related to the degree of enamel mineralization. Therefore, each pseudo color in these images signifies a certain amount of phosphate in accordance with the adjacent standardization bars. The yellow tint designates high mineral content in the outermost enamel layer and the unaffected inside enamel layer, which represents the end of the lesion. Whereas the body of the lesion looks brown in color due to a severe reduction in PO_4^{3-} amount in this area **(**Figure 1FA-GC). A slight reduction in lesion depth was observed after remineralization **(**double-ended arrows in Figure 1D and FD-KA), besides, clear evidence of remineralization of the subsurface lesions and ISLs followed treatment with nHA-containing dentifrice (arrows in Figure 1IC-KA, FC-KA). Data analysis verified that there were no significant differences ($p>0.05$) among the groups before and after treatment with CPP-ACP with respect to PO_4^{3-} intensity and enamel crystallinity in subsurface lesion and ISL zones (Figure 2A and B). Analysis demonstrated that nHA-containing dentifrice significantly ($p<0.05$) promoted mineral gain in the enamel subsurface lesion and ISL areas (Figure 2C). However, no significant differences were noticed among the treatment groups regarding enamel crystallinity (Figure 2D). The correlated influence of CPP-ACP and nHA-containing dentifrice on the subsurface lesion and ISL areas is shown in Figure 2E, F to detect distinguished differences in their performance. The only significant difference was attained in respect to mineral gain in the body of lesions treated by dentifrice compared to the counterpart treated by CPP-ACP (Figure 2E). The statistical analysis showed no differences in the exerted effect of both products on mineral gain in ISL and on the degree of crystallinity in ISL and subsurface lesion zones (Figure 2E, F). The rate of changes in phosphate intensity and FWHM value in ISL and subsurface lesion regions after treatment with remineralization agents are shown in Table 1. The change in mineral gain proves that CPP-ACP exerted a minor remineralizing effect on ISL of the lesion (+9.2%). While mineral loss from the subsurface layer has been detected after CPP-ACP application (-8.1%). nHA-containing dentifrice exhibits an important tendency to remineralize subsurface lesions (+96.6%) and, to a lesser extent, ISL zone (+39.66).

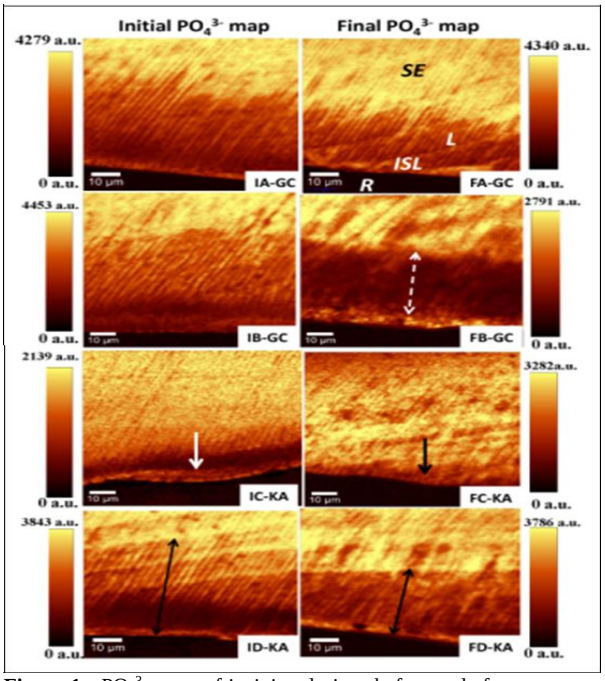

Figure 1: PO_4^{3-} maps of incipient lesions before and after treatment with remineraling agents. Indicative standardization bars on both sides demonstrate max. and min. values of PO_4^{3-} peak intensity at 960 cm^{-1}. **GC:** CPP-ACP tooth mousse. **KA:** KAREX (nHA containing-dentifrice). **I:** Initial. **F:** Final. Signs on Image (FA-GC) are valid for all images: **SE:** Sound Enamel, **L:** Lesion, **ISL:** Intact Surface Layer, **R:** Resin.

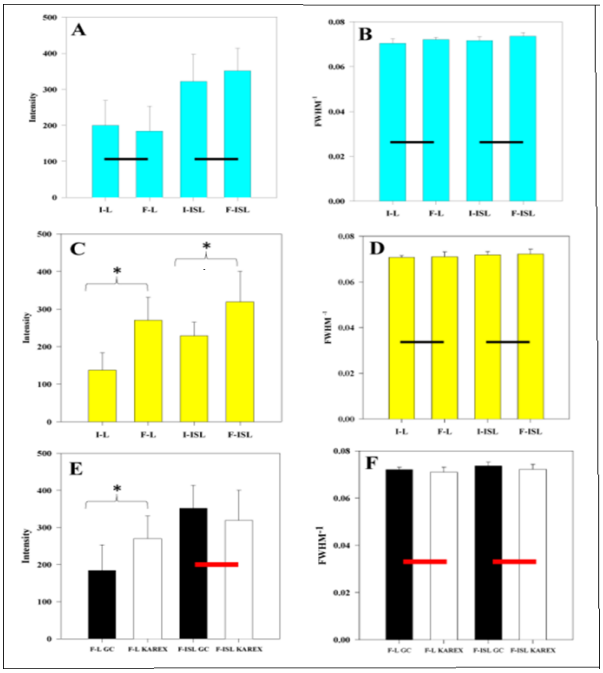

Figure 2: The mean and standard deviation (SD) were plotted to show: effect of: **(A)** CPP-ACP & **(C)** KAREX on PO_4^{3-} peak intensity at 960 cm^{-1}. Effect of: **(B)** CPP-ACP & **(D)** KAREX on enamel crystallinity represented by $FWHM^{-1}$ values. **(E&F)** Associating effect of CPP-ACP & KAREX on PO_4^{3-} peak intensity & $FWHM^{-1}$ respectively. The treatment effects of both agents were tested in (**L)** Lesion zone & (**ISL)** Intact Surface Lesion zone consecutively. **I:** Initial. **F:** Final. **GC:** CPP-ACP tooth mousse. **KAREX:** nHA containing-dentifrice. Asterisk (*) recurrently indicates a significant difference (p<0.05) between the two groups. Horizontal bar indicates no significant difference (p>0.05) among the groups linked by it.

The table shows negative changes in FWHM value in ISL and subsurface lesion regions after treatment with remineralization agents, underlining an improvement in enamel crystallinity. CPP-ACP had a better effect on enamel texture than nHA-containing dentifrice.

DISCUSSION

Non-invasive management of non-cavitated caries lesions represents an essential goal of modern dentistry, involving remineralization systems to repair the enamel with bioavailable calcium & phosphate ions. The CPP acts as a delivery vehicle to co-localize bioavailable calcium and phosphate ions at the tooth surface. It can adapt its conformation to a wide range of surfaces, including the amorphous phases, thereby binding spontaneously forming clusters of calcium phosphate ions in a metastable solution, preventing their growth to the critical size required for nucleation [3]. The mechanism of action of CPP-ACP needs to be considered at a location inside the subsurface lesion, as well as at the surface of the lesion. The CPP-ACP has been determined to be amorphous electroneutral nanocomplexes with a hydrodynamic radius of 1.53±0.04 nm [20]. From the size and electroneutrality of the nanocomplexes, it would be predictable that they would enter the porosities of demineralized enamel and diffuse down concentrated ingredients into the lesion body. The released calcium and phosphate ions at the enamel surface will participate in a variety of equilibria to form a range of calcium phosphate species, depending on the pH [21]. The process of diffusion into a subsurface lesion must be an overall electro-neutral process. Therefore, diffusion potential into an enamel subsurface lesion can be characterized by the active ingredients of the neutral ion pair, $CaHPO_4^0$, diffusing down its ingredients into the lesion [6,21]. Once present in the enamel subsurface lesion, the CPP-ACP would release the weakly bound calcium and phosphate ions [20,22], which would then deposit into crystal voids, promoting crystal growth at the hydroxyapatite (001) plane or along the c-axis, resulting in strengthening and widening of enamel rods [23]. The CPPs have a high binding affinity for apatite [24]; hence, on entering the lesion, the CPPs would bind to the more thermodynamically favored surface of the apatite crystal face. Therefore, the release of bioavailable ions would be thermodynamically driven. This theory could explain the negative change in FWHM value illustrated by Table 1, where the reduced width of the phosphate band after treatment with CPP-ACP reflects a minor enhancement in enamel crystalline configuration. However, supersaturation with respect to HA, in which no spontaneous precipitation occurs, can't be expected to exist for a long period. After the contact between enamel apatite and the supersaturated solution, the mineral formation by the growth of existing, partly demineralized crystals largely reduces the supersaturation level in a way that

spontaneous nucleation is unlikely to occur [25,26]. This could explicate the limited influence of remineralizing agents on enamel crystallinity reconstitution (Figure 2B and D). Since the degree of hard tissue mineralization is directly related to phosphate sum intensity [27]; Raman phosphate maps can show that CPP-ACP has a slight remineralization effect on ISL. Whereas the dashed line in image (FB-GC, Figure 1) depicts an undesirable change in the mineral content of the subsurface layer (-8.1%). Previous studies [28,29] indicated that mineral deposition is most easily formed superficially, making the surface layer of the lesion appear harder and glossy. While the subsurface porous, demineralized part of the lesion remains unaltered, explaining why a proper remineralization of the body of the lesion rarely occurs (Figure 1, FB-GC). Larsen [30] (one of the pioneers in remineralization research) tested the results of enamel powder produced from intact teeth, suspended in remineralizing solutions. A reduction in pH value was observed during apatite formation because almost all phosphate in the aqueous phase prior to precipitation is in the form of H_3PO_4, $H_2PO_4^-$ and HPO_4^{2-} while the same phosphate after the uptake in the apatite is mostly in the PO_4^{3-} form according to the following reaction [25]:

$$10\ Ca^{+2} + 3HPO_4^{2-} + 3H_2PO_4^- + 2\ H_2O \longrightarrow 2Ca_5(PO_4)_3OH + 11\ H^+$$

The released H^+ ions cause the pH to drop. The simultaneous pH decrease inhibits precipitation of calcium and phosphate in the aqueous phase, but it has a solubilizing effect on the enamel mineral [31]. This could explain the reduced amount of minerals that has been detected in subsurface lesions being treated with CPP-ACP. The impaired effect of CPP-ACP reported in this study is in accordance with previous observations stated in several clinical studies [32-35], conducted to detect the remineralizing effect of CPP-ACP cream *in vivo*. They failed to show any significant benefit of this product in reversing WSLs noninvasively. Even though the absence of effects might be due to the ineffectiveness of this agent or insufficient sample sizes to detect significant differences. Thus, further research to verify the efficacy of this combined therapy would be beneficial. Nevertheless, the current findings seem to be inconsistent with those reported in former *in vitro* studies. The tested effect of the ion composition of CPP-ACP solutions on subsurface enamel lesion restoration [6,21], has demonstrated that CPP-ACP can promote remineralization of subsurface enamel lesions. The fact that they tested the effect of CPP-ACP solutions, while our current study has examined the remineralizing effect of CPP-ACP topical cream assigned to in-office/at-home application, could stand behind this discrepancy. Solutions are less viscous than creams; thus, better diffusion is predictable since viscosity is inversely proportional to diffusivity [36]. The surface chemical properties and morphological structure of nHA, combined with its chemical and physical similarity with natural enamel, have been claimed to play an important role in the remineralization of early caries lesions [12]. Many factors increased the potential of nHA to fill up defects and micropores on demineralized enamel, such as increased surface area, increased proportion of atomicity, and solubility properties of nHA [37]. When nHA penetrates the enamel pores, it will act as a template in the precipitation process. Thereby, it will constantly attract large amounts of Ca^{2+} and PO_4^{3-} from the remineralization solution to the enamel surface to fill the vacant positions of the enamel crystals. This in turn will reinforce crystal integrity and growth [10]. Consequently, the deposition of nHA on the outer enamel layer would probably block surface pores and restrict diffusion into subsurface lesions over the short-term remineralization. However, nHA is progressively transferred from the new sediment apatite coating inward. Finally, it is precipitated in the body of the subsurface lesion in the long-term remineralization [14,38]. It is noteworthy that nHA-containing dentifrice has been tested in this study. Its influence was found to be superior to the CPP-ACP paste regarding subsurface lesions remineralization (Figure 2E). Phosphate maps in Figure 1A and B affirm that CPP-ACP has a limited remineralizing influence on the body of the lesion. Contrary to nHA-containing dentifrice (Figure 1C and D), which has triggered a patent remineralization of subsurface lesions (+96.6%). Viscosity tests confirmed that CPP-ACP cream is more viscous than nHA-containing dentifrice (2665 P versus 1846.65 P, respectively). As viscosity is inversely proportional to the diffusivity, the diffusion process represents a limiting factor in the subsurface lesion remineralization (28, 36). Consequently, nHA-containing dentifrice produced more profound remineralization of subsurface lesions thanks to its lower viscosity and thus better ionic diffusion rates. Therefore, viscosity of the tested pastes could be considered as a key factor that might explain the discrepancy in their treatment effect. Figure 2 (A and C) describes the quantity of phosphate components and confirms that the outermost layers of teeth used to assess nHA-containing dentifrice capability were basically less mineralized and thus more porous than surface layers of teeth implicated in testing CPP-ACP efficacy. For mineral deposition to occur within the body of the lesion, calcium and phosphate ions must first penetrate the enamel surface. Therefore, the presence of a relatively mineralized and charged surface layer covering severely porous underlying enamel may result in restricted permeability of the ions and ion pairs necessary for mineral formation and subsequent underlying layer remineralization [30]. In addition, the large variations in enamel chemical composition with respect to concentrated ingredients of

specific mineral ions are therefore likely to result in large local variations in rates of both demineralization and remineralization [39-41]. Remineralization systems represent a major advance in the non-invasive treatment of incipient caries lesions. However, further studies of the biomimetic molecules involved in calcium and phosphate stabilization and nucleation may provide further improvements in the development of novel remineralization technologies as BioMin® toothpaste is newly innovated bioactive material made with extra fine particles that bond to the tooth enamel to strengthen and protect it through the formation of fluorapatite crystals.

Conclusion

The CPP-ACP effect is confined to ISL, making it a less permeable layer. These results are inconsistent with the proposed anticariogenic mechanism of the CPP, which is enhancement of remineralization through the localization of ACP at the tooth surface. In addition, nHA-containing dentifrice has the potential to remineralize the whole lesion consistently. The study indicated a lack of reliable evidence supporting the effectiveness of remineralizing agents in WSL treatment. Likewise, de novo formation of crystals in the body of the lesions remains an entirely unsolved problem. Largely, the present study points out that the consistency of remineralizing material with the enamel surface layer permeability represents serious obstacles to remineralization.

Ethical considerations

Samples were collected with patients' informed consent and approval of the local ethical research committee (Comité de protection des personnes-CPP Sud Mediterranee IV, process No. 2017-2907) before usage. All procedures were carried out in accordance with relevant guidelines and regulations.

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Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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