

SPECIAL ISSUE RESEARCH ARTICLE

The application of casein phosphopeptide and amorphous calcium phosphate with fluoride (CPP-ACPF) for restoring mineral loss after dental bleaching with hydrogen or carbamide peroxide: An in vitro study[☆]

Carmen Llana^{a,*}, Irene Esteve^a, Francisco Javier Rodríguez-Lozano^b, Leopoldo Forner^a

^a Department of Stomatology, Universitat de Valencia, Spain

^b Unit of Special Care in Dentistry and Cell Therapy and Hematopoietic Transplant Unit, Internal Medicine Department, IMIB-Arrixaca, University of Murcia, Murcia, Spain

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ABSTRACT

Objective: To conduct an in vitro evaluation of the effect of casein-phosphopeptide and amorphous calcium phosphate with fluoride [CPP-ACPF] upon the calcium [Ca] and phosphorus [P] composition and morphology of dental enamel and dentin after the application of two bleaching agents: 37.5% hydrogen peroxide [HP] and 35% carbamide peroxide [CP].

Materials and methods: The crowns of 40 extracted human teeth were divided into four groups (n = 10 each). The crowns were sectioned along the cervical-incisal axis, and each half was embedded in acrylic resin, leaving a window 3 mm in diameter to explore the enamel or dentin (according to the study group involved). Groups 1a and 1b corresponded to the two halves belonging to the same tooth and were treated with HP applied to enamel three times for 10 min. Afterwards, CPP-ACPF was applied in group 1b for five minutes. The same procedure was carried out in groups 2a and 2b, but application was done on dentin. Groups 3a and 3b were treated with CP applied to enamel once for 30 min. Posteriorly, CPP-ACPF was applied in group 3b for five minutes. Groups 4a and 4b were treated in the same way as groups 3a and 3b, but application was done on dentin. The morphological changes were evaluated using confocal laser scanning microscopy (CLSM), and the changes in Ca and P composition were assessed by environmental scanning electron microscopy coupled to a microanalytical system (ESEM + EDX). Comparisons between groups were made using the Kruskal–Wallis test, and Duncan test was applied for two-by-two comparisons, with a significance level of $p < 0.05$.

Results: Both HP and CP produced similar changes in enamel morphology and produced no changes in dentin. Groups treated with CPP-ACPF after bleaching improved the percentage of Ca and P. Statistical significance was reached only in the case of the percentage of Ca in enamel (group 1b versus group 1a, $p = 0.01$) (group 3b versus group 3a, $p = 0.03$).

Conclusions: The application of CCP-ACPF for five minutes on the enamel surface after tooth bleaching is effective in restoring the Ca lost as a result of the bleaching process, while their effect in dentin could not be demonstrated.

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1. Introduction

The current dental bleaching agents are mainly based on hydrogen peroxide (HP) or carbamide peroxide (CP), which act by oxidizing the large chromophore molecules responsible for enamel

and dentin discoloration. Hydrogen peroxide decomposes into oxygen and free radicals which in turn oxidize the staining molecules, giving rise to colorless shorter-chain molecules that can be transformed into carbon dioxide and water at the end of the process (1). Enamel and dentin permeability, and the low molecular weight of HP, facilitate penetration of the latter into the hard dental tissues (Haywood, 1990; Chen et al., 1993).

Some studies have described changes in the morphology, chemical composition and micro-hardness of enamel and dentin that can alter their biomechanical properties (Sa et al., 2013; Goldberg et al.,

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* Corresponding author.

E-mail address: llena@uv.es (C. Llana).

2010; Forner et al., 2009). Likewise, changes have been reported in the organic component of the mineralized dental tissues (Bitter, 1992; Severcan et al., 2008). Studies on changes in the chemical composition of the mineral component of enamel and dentin as a result of exposure to different whitening or bleaching agents have also been made, with heterogeneous results. While authors, such as Cakir et al., have observed no changes using products based on low-concentration CP (Cakir et al., 2011), Soares et al. reported Ca and P loss in enamel after using CP at a concentration of 10–16% (Soares et al., 2013). Maleknejad et al. in turn recorded changes in Ca and P composition in enamel and dentin after bleaching action with high-concentration HP and CP (1 Maleknejad et al., 2012).

Products based on casein-phosphopeptide and amorphous calcium phosphate (CPP-ACP) have been shown to be able to remineralize the tooth in vitro (Gjorgievska et al., 2013). Proportion of Ca lost in the demineralization process characterizing dental caries is reduced in the presence of CPP-ACP, with rapid restoration of a normal Ca concentration (Yamaguchi et al., 2006). Casein exerts a direct buffering effect against dental plaque acidity thanks to amino acid release, or an indirect effect by acting upon bacterial metabolism (Rose, 2000). Some studies have found that its application to whitened or eroded teeth increases enamel microhardness and reduces its roughness (da Costa Soares et al., 2013; de Vasconcelos et al., 2012; Khoroushi et al., 2011; Heshmat et al., 2014; Heshmat et al., 2016; Penumatsa et al., 2015). However, as far as we know, there is no information on the effect of CPP-ACP with fluoride (CPP-ACPF) upon Ca and P concentration in enamel and dentin after bleaching with products destined for professional use.

The present in vitro study evaluated the effect of a paste based on CPP-ACPF upon the changes in Ca and P composition and on enamel and dentin morphology after the application of two professional dental bleaching products: Pola Office+ (37.5% HP) and Pola Day CP (35% CP), both at neutral pH. The study hypothesis was that the use of a remineralizing agent after dental bleaching with HP or CP does not modify the mineral composition of either the enamel or dentin.

2. Material and methods

Human teeth (maxillary incisors and canines) extracted for different therapeutic reasons were used for the study, following approval from the Research Ethics Committee of the University of Valencia -Valencia, Spain- (Ref.: H1391449402592).

The teeth were carefully examined under $\times 10$ magnification, discarding those with cracks, caries or structural enamel defects. The final study sample consisted of 40 teeth which were cleaned of tartar and stored in 0.1% thymol for five days.

2.1. Sample preparation

The teeth were then sectioned 2 mm from the amelodentinal junction using a water-refrigerated diamond disc (Isomet; Buehler, Lake Bluff, IL, USA). The roots were discarded. The crowns were randomized to four groups ($n = 10$ each), and each crown was sectioned along the cervical-incisal axis with a diamond disc. The resulting halves were embedded in acrylic resin (Buehler, Lake Bluff, IL, USA), leaving a window (Fig. 1) to explore the enamel or dentin (according to the study group). The dental surface was leveled with water-refrigerated carborundum discs –320, 600 and 1200 grit- (Buehler, Lake Bluff, IL, USA) and polished with a 8000 grit diamond disc (3M TM Dental Products, St. Paul, MN, USA) for two minutes. We then applied EDTA for 30 s and immersed the samples in an ultrasound bath for 10 min. The samples were stored in artificial saliva composed of 150 mmol/l KCl, 1.5 mmol/l CaCl₂ and

Table 1
Study groups.

1a	HP 37,5% 3 × 10 min enamel
2a	HP 37,5% 3 × 10 min dentin
1b	HP 37,5% 3 × 10 min +CPP-ACFP 5 min enamel
2b	HP 37,5% 3 × 10 min +CPP-ACFP 5 min dentin
3a	CP 35% 30 min enamel
4a	CP 35% 30 min dentin
3b	CP 35% 30 min +CPP-ACFP 5 min enamel
4b	CP 35% 30 min +CPP-ACFP 5 min dentin

HP: hydrogen peroxide, CP: carbamide peroxide, CPP-ACFP: Mi paste plus, min: minute.

0.9 mmol/l KH₂PO₄ per 100 ml of bidistilled water at 37 °C, until use. All samples were washed for 30 s with deionized water before bleaching treatment.

2.2. Application of the bleaching and remineralizing agents

The samples belonging to groups 1a and 1b corresponded to the halves from the same tooth, and underwent three 10-min treatments with 37.5% HP (Pola Office+, SDI, Victoria, Australia) applied to the enamel to a thickness of about 1 mm. Between applications, the material was removed with an aspirator, followed by washing for 30 s with deionized water. Then, in group 1b we applied CPP-ACPF paste (MI Paste plus, GC Corporation, Tokyo, Japan) to a thickness of about 1 mm for 5 min, as recommended by the manufacturer, followed by removal with deionized water. The same procedure was carried out in groups 2a and 2b, but application was done on dentin.

In groups 3a and 3b, Pola Day CP -35% CP- (SDI, Victoria, Australia) was applied to the enamel to a thickness of about 1 mm, for 30 min. The gel was then removed by aspiration, followed by washing with deionized water for 30 s. Then, in group 3b we applied CPP-ACPF paste to a thickness of about 1 mm for five minutes, followed by removal with deionized water. The same procedure was carried out in groups 4a and 4b, but application was done on dentin.

Table 1 summarizes the treatment protocol used in each group. During the period of application of the bleaching gel, the samples were covered with transparent film to retain moisture, and, after treatment and washing, they were kept in deionized water for 24 h, followed by morphological study and the analysis of Ca and P composition.

2.3. Evaluation of the morphological changes

The samples were placed on a dish filled with distilled water and examined under $\times 40$ magnification with immersion oil using a FV1000 confocal microscope (Olympus, Melville, USA). Visualization was made under reflection mode with illumination provided by a HeNe laser at a wavelength of 633 nm.

The orientation of the enamel prisms is not the same over the entire enamel surface. We therefore selected areas in which a full cross-section could be observed. Once the area was located, a general view was recorded, followed by using a $\times 3$ zoom, focused on an optimum clear zone of the general image. In the case of the dentin samples we sought an image affording a longitudinal sectional view of the tubules and amelodentinal junction.

2.4. Study of the proportions of Ca and P

The samples were examined under an ESEM XL30 environmental scanning electron microscope (Olympus, C, Melville, USA), and the proportions of Ca and P were determined on a semiquantitative

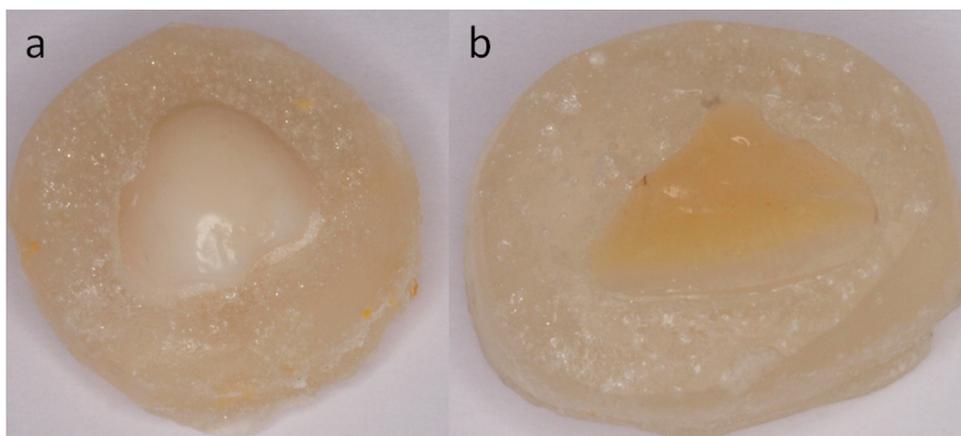


Fig. 1. Sample preparation.

a: enamel, b: dentin.

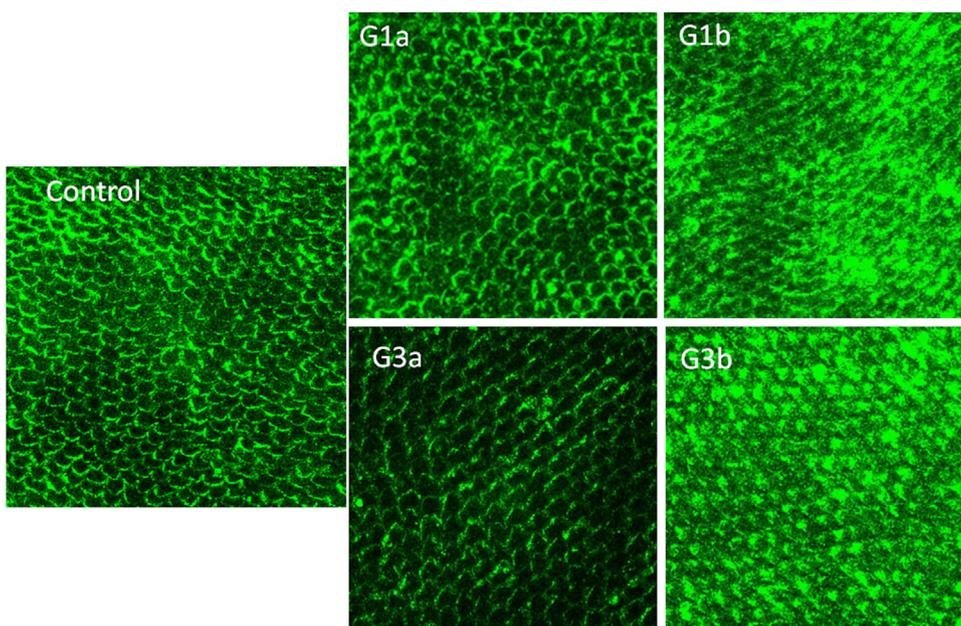


Fig. 2. Morphological changes in enamel.

Control: appearance of untreated enamel. Group 1a: appearance of the enamel after three 10-min applications of Pola Office+. Group 1b: appearance of the enamel after three 10-min applications of HP to Pola Office+ and Mi Paste Plus applied for five minutes. Group 3a: appearance of the enamel after one 30-min application of Pola Day CP. Group 3b: appearance of the enamel after one 30-min application of Pola Day CP and Mi Paste Plus applied for five minutes.

basis with a microanalytical system -EDX- (Genesis, Weiterstadt, Germany). The following operating parameters were used: working distance 10 mm; acceleration voltage 20 kV; illumination 100 μ A and an acquisition time of 50 s. For the microanalysis, we located the central zone of the sample and performed three measurements: one of the central zone and another two at two points equidistant to the first, along the diagonal of each sample. The proportions of Ca and P in the enamel and dentin were recorded in all the groups and the mean of the three measurements was calculated.

2.5. Statistical analysis

The Kolmogorov–Smirnov test confirmed the non-normal distribution of the data referred to Ca and P composition. Comparisons were thus made between groups using the Kruskal–Wallis test, and the Duncan test was performed for two-by-two comparisons. Statistical significance was considered for $p < 0.05$ in all cases.

3. Results

3.1. Morphological analysis (CLSM)

Correctly conformed enamel prisms are transparent to laser light, while less structured areas of the inter-prism spaces reflect the light of the confocal laser scanning microscope (CLSM). The combination of light reflection and transmission produces the honeycomb pattern characteristic of enamel prisms. The images obtained after treatment with HP and CP showed a similar pattern, with the observation of irregularities in the heads of the enamel prisms. After the application of CPP-ACPF, we observed amorphous material deposits on the enamel surface in both the HP and CP treated samples (Fig. 2).

The appearance of the dentin treated with HP or CP was likewise similar. The amelodentinal junction was visualized, together with branching of the dentinal tubules penetrating the enamel layer-being this indicative of an intact structure of the zone (Fig. 3).

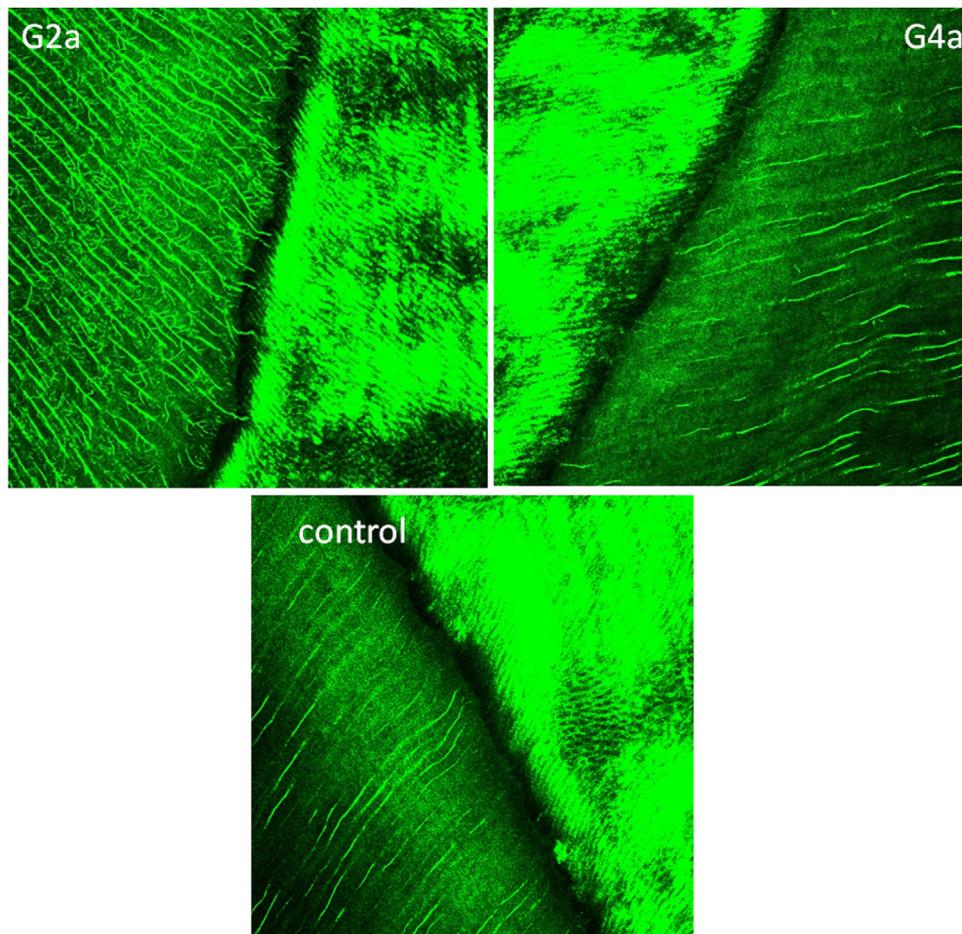


Fig. 3. Morphological changes in dentin.

Control: appearance of untreated dentin. Group 2a: appearance of the dentin after three 10-min applications of Pola Office+. Group 4a: appearance of the dentin after one 30-min application of Pola Day CP.

3.2. Analysis of Ca and P composition

The percentage composition of Ca and P in enamel was slightly lower in the subgroups treated with HP (1a) when compared to the treated with CP (3a), but without significant statistical differences. Following the application of CPP-ACPF, the percentage of Ca (CaKT) in group 1b (HP + CPP-ACPF) was significantly greater than in group 1a -HP- ($p = 0.01$). The same applied to subgroup 3b (CP + CPP-ACPF) with respect to subgroup 3a -CP- ($p = 0.03$). The weight percentage of Ca (CaKWt), was higher in subgroups treated with CPP-ACPF, but differences were not statistically significant ($p > 0.05$). The same was found in weight and percentage of P (Fig. 4).

In dentin, although the composition in weight and percentage of Ca and P was slightly greater in the subgroups where CPP-ACPF was applied after bleaching (2b/4b), the differences with respect to the groups in which only the HP or CP was applied (2a/4a) failed to reach statistical significance ($p > 0.05$), as represented in Fig. 5.

4. Discussion

Few studies have evaluated the changes induced by bleaching agents related to dentin morphology and mineral composition. It is important to study such changes, especially in view of the possible consequences of dental bleaching after a root canal treatment, where the bleaching agent comes into direct contact with the dentin. Confocal laser scanning microscopy (CLSM) was used to visualize the surface and sub-surface changes because it offers

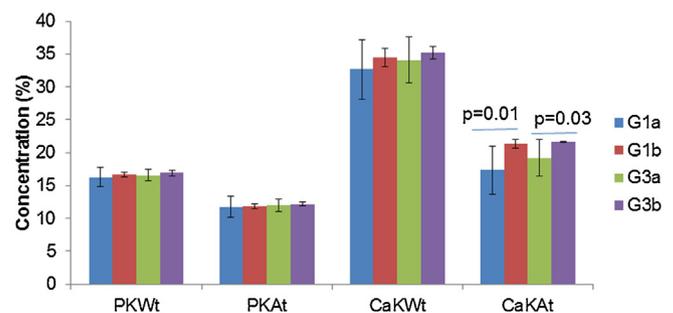


Fig. 4. Changes in Ca and P composition in enamel.

Group 1a: Pola Office+. Group 1b: HP (Pola Office+) + Mi Paste Plus. Group 3a: Pola Day CP. Group 3b: Pola Day CP + Mi Paste Plus. PKWt: percentage weight of P, PKAt: percentage of P atoms, CaKWt: percentage weight of Ca, CaKAt: percentage of Ca atoms.

the advantage of not requiring additional specimen treatment, and the hard dental tissues are kept under moist conditions and can be used for posterior analyses (Markovic et al., 2007; Gotz et al., 2007; Llana et al., 2018). Standard image acquisition conditions were maintained to allow comparisons at a later stage.

No morphological changes were observed either in the dentin or at the amelodentinal junction as a result of the application of 37.5% HP (group 1a) or 35% CP (group 3a). These findings were previously described by our group (Llana et al., 2018). Some studies presented an increment of the dentinal tubules diameter after dentin bleach-

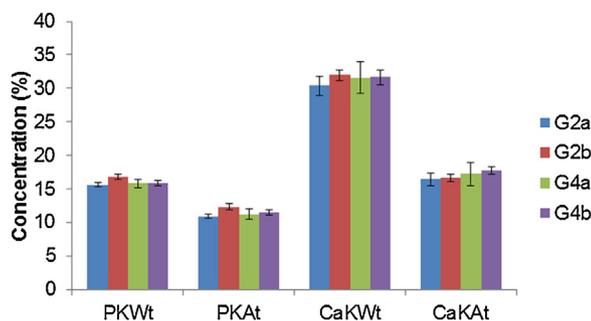


Fig. 5. Changes in Ca and P composition in dentin. Group 2a: Pola Office+. Group 2b: HP (Pola Office+) + Mi Paste Plus. Group 4a: Pola Day CP. Group 4b: Pola Day CP + Mi Paste Plus. PKWt: percentage weight of P, PKAt: percentage of P atoms, CaKWt: percentage weight of Ca, CaKAt: percentage of Ca atoms.

ing -with HO and with CP- (Joiner, 2007), nevertheless results are not comparable, because they have been performed with a SEM and we used a CLSM.

ESEM/EDAX study main advantage is to provide an accurate and non-destructive analysis of the dentin and enamel composition. In dental research, levels of Ca and P in enamel and dentin are a good indicator of the demineralization/remineralization processes (Cakir et al., 2011). Some authors described an increase of voids, loss of the tissue structure and some other irregularities after using high-concentrated bleaching agents (Forner et al., 2009; Coceska et al., 2016; Llana et al., 2018). The present study shows this findings too, in the HP and in the CP groups. After CPP-ACPF application, we observed amorphous material deposits on the enamel surface in both the HP and CP treated samples. These results have been previously studied through scanning electronic microscopy -SEM- (Poggio et al., 2016), where a thin and homogeneous layer was described after CPP-ACPF application.

In the present study, the composition of Ca y P (in terms of weight percentage and elemental composition) were lower in the groups treated with HP versus the CP group, mainly for enamel Ca, probably because of the higher concentration of the active agent (H_2O_2), three times higher in HP than in CP (but differences were not significant); finding that confirm previous results obtained by our group in a previous study (Llana et al., 2018), where also mineral loss was compared with paired non-treated controls. In this last case, treated cases did not show significant decreases. For this reason, non-treated control was not included in the present study. Many authors refer a mineral loss in enamel and dentin when bleaching agents are applied, but there are not an agreement on the level of demineralization (Lee et al., 2006; Cakir et al., 2011). Lee et al. (2006) found an enamel mineral loss (after 30% HP application) similar to those produced after a soft drink.

The oxidation–reduction processes caused by bleaching agents give rise to deconstruct the organic and inorganic matrix of the enamel and dentin (Soldani et al., 2010). In combination with the physiological components present in saliva, the use of different remineralizing agents have been proposed to help restore the mineral component of enamel (Heshmat et al., 2016). However, the remineralizing effect of saliva exerts protective action that reverts the effect of the bleaching agents. As a result, the clinical relevance of mineral loss caused by bleaching agents may be limited (Metz et al., 2007). Nevertheless, different products have been proposed in order to facilitate remineralization. Fluoride gives controversial results. While some authors have reported improved enamel properties after applying fluoridated substances to the bleached enamel surface (Attin et al., 1997; Borges et al., 2009), others have observed no beneficial effects in terms of either improved properties or increased mineral component, neither providing fluoridated

products after bleaching nor using a bleaching agent combined with fluoride (Burgmaier et al., 2002; Tschoppe et al., 2009). In the present study, fluoride levels were not evaluated, being a limitation.

Amorphous calcium phosphate (ACP) stabilized with casein-phosphopeptides (CPP) affords an excellent bioavailable Ca and P reserve (Cochrane et al., 2010). In the present study, a remineralizing treatment with a CPP-ACPF product, conditioned a significant increase of Ca levels in enamel in both group (treated with HP and treated with CP), nevertheless, these changes were not significant in dentin. This could be explained by the different demineralization/remineralization processes in dentin and enamel (Chien et al., 2016). Other studies did not find an increase of Ca and P in dental mineralized tissues after CPP-ACP treatment of previously bleached enamel (with 38% HP). We used CCP-ACPF. Small fluoride quantities can change Ca fixation over the hydroxyapatite structure (Christoffersen et al., 1988). Nevertheless P levels did not show differences before and after CPP-ACPF application, as previously described (Coceska et al., 2016).

In the present study we rejected the hypothesis that the use of a remineralizing agent after dental bleaching with HP or CP does not modify the mineral composition of enamel, since after a single five-minute application of a CPP-ACPF-based product we not only observed the presence of precipitates, which is consistent with the findings of other studies (de Abreu et al., 2011; de Vasconcelos et al., 2012; Gjorgievska et al., 2013), but also recorded a significant increase in the percentage of Ca, as referred by Bayrak et al. (2009), Klaric et al. (2015) and Coceska et al. (2016).

From the clinical perspective, this mineral increment could also imply decreased dental sensitivity thanks to the depositing of mineral in the enamel crystals, thereby affording improved patient safety and comfort, as evidenced in a recent clinical trial (Alexandrino et al., 2017).

Freshly extracted human teeth were used in this study. In order to secure maximum sample homogeneity in terms of their mineral composition, both the teeth in the groups treated with the bleaching agent only and the teeth in the groups treated with the bleaching agent plus CPP-ACPF (as remineralizing product) were sectioned into two halves. Each half was assigned to one of two subgroups (a or b), following the methodology proposed by Markovic et al. (2007), and which had already been used in one of our previous studies (Llana et al., 2018).

Accepting the limitations inherent to in vitro studies, and based on the results obtained in our study, it can be concluded that the application of Mi Paste Plus for five minutes on the enamel surface after tooth bleaching treatment using high-concentration products is effective in restoring the Ca lost as a result of the bleaching process, while their effect in dentin could not be demonstrated.

Ethical statement

Human teeth (maxillary incisors and canines) extracted for different therapeutic reasons were used for the study, following approval from the Research Ethics Committee of the University of Valencia -Valencia, Spain- (Ref.: H1391449402592).

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