Confocal microscopy investigation of enamel subsurface structure following bleaching

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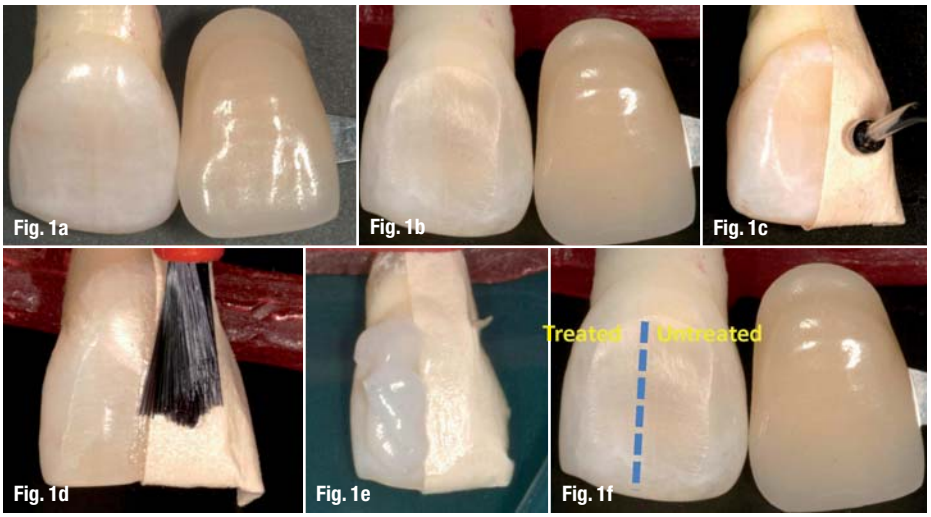


Figs. 1a–f_Pre-op view of one of the incisors selected for the study (a). Flattened area (≈ 4 x 5 mm) created by serial polishing with sandpaper up to 1,200 grit (b). A piece of water-resistant tape is applied to one-half of the tooth and burnished (c). The edge of the tape is sealed by painting it with a transparent nail varnish (d). Bleaching gel is applied to the exposed area (e). Diagrammatic representation of the control and treated areas (f).

_Tooth-whitening procedures have been associated with morphological changes in the enamel surface. As early as 1993, Shannon *et al.* reported significant surface topographic alterations in enamel slabs that had been treated with bleaching solutions for four weeks.[1] These findings were confirmed in another study with 30 % H_2O_2 mixed with PBS.[2] Teeth that were bleached *in vivo* with 35 % carbamide peroxide lost the aprismatic enamel layer and the damage was not repaired after 90 days.[3] A confocal laser scanning microscopic study evaluating the micro-roughness of enamel surfaces after bleaching procedures with 10 and 16 % carbamide peroxide found roughness to be significantly higher than in control surfaces.[4]

However, another study reported no or minimal changes in the enamel surface after bleaching. Leonard *et al.* evaluated casts made from impressions of teeth bleached with 10 % carbamide peroxide for eight to ten hours per day for 14 days, and found no or minimal changes in the enamel surface.[5] Their findings may be explained by the limits of the methodology, in which there was inadequate reproduction of the minor enamel alterations in the impression. Other *in vitro* SEM studies published recently have also revealed no enamel irregularities after bleaching.[6,7]

In general, recent literature supports the theory that a high concentration of carbamide peroxide is detrimental to enamel surface integrity, while low concentrations were found to produce no changes.[8–11] A clinical implication of these findings may be that the teeth are more susceptible to extrinsic discolouration after bleaching due to increased surface roughness.

The changes may be deeper than superficial. Using infrared spectroscopic analysis, Oltu and Gürgan reported that *in vitro* treatment of extracted teeth with 35 % carbamide peroxide for 30 minutes per day for four days changed the inorganic composition of the enamel, whereas 10 and 16 % concentrations did not.[12] Cavalli *et al.* also demonstrated that bleached dentine could lose inorganic components, resulting in ultrastructural alterations.[13]

Studies have demonstrated a dynamic process of demineralisation of human enamel intra-orally.[14] In these studies, the existence of subsurface enamel pores, which increase and decrease in size in relation to this de- and remineralisation process, has been demonstrated. Amorphous calcium phosphate (ACP) has been shown to shift the de-/remineralisation balance towards remineralisation, resulting in a decrease in size and/or number of pores.[15] It has been theorised that bleaching creates subsurface

Table I_Bleaching agents used in the study.

Bleaching agent	Main ingredient and concentration	Manufacturer
Nite White Excel 3 with ACP	16 % hydrogen peroxide (+ ACP)	Discus Dental
Opalescence PF	10 % carbamide peroxide (+ fluoride and potassium nitrate)	Ultradent

Table I

pores and that this is a cause of the transient sensitivity experienced by some people when they whiten their teeth.

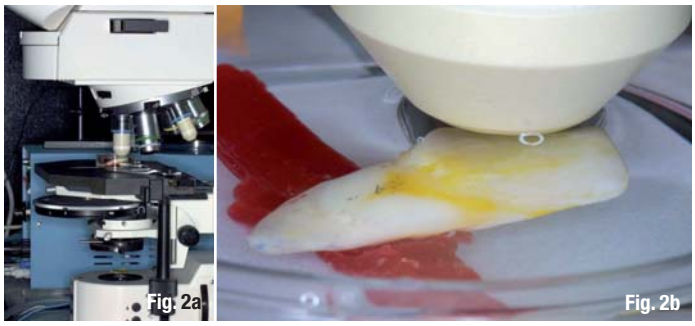
Recently, some companies have added ACP to their formulations. Claims have been made that the addition of ACP reduces tooth sensitivity by decreasing the size of these pores.[16] In addition, by filling minor defects within the enamel, the addition of ACP creates an enamel surface that is smoother and more lustrous. However, in an *in vitro* study on bovine incisors, no supporting influence of fluoride-containing bleaching gels on remineralisation was observed.[17]

The purpose of this study was to evaluate enamel subsurface structure following application of two bleaching agents to extracted incisor teeth using confocal microscopy.

_Materials and method

A flattened area (≈ 4 x 5 mm) was created on the labial surface of extracted central and lateral incisor teeth (n = 10) by serial polishing with SiC sandpaper of up to 1,200 grit (Figs. 1a & b). It is true that confocal microscopy holds greater advantages for samples that cannot be polished to a flat surface. In our case, the flattened surface helped in orienting the area of interest as a plane-parallel object held perpendicular to the optic axis for sharper image.

The teeth were ultrasonically cleaned with distilled water to remove debris. A piece of water-resistant tape was applied to one-half of the tooth and burnished (Fig. 1c). The edge of the tape was sealed by painting it with a transparent nail varnish (Fig. 1d). The exposed area was randomly assigned to one of two groups. The first group (ACP group) was treated with Nite White Excel 3 with ACP (Discus Dental). The second group (OP group) was treated with Opalescence PF 10 % (Ultradent). The untreated control for both groups was the area underneath the tape, allowing each tooth to serve as internal control. The composition of the whitening products is described in Table I.

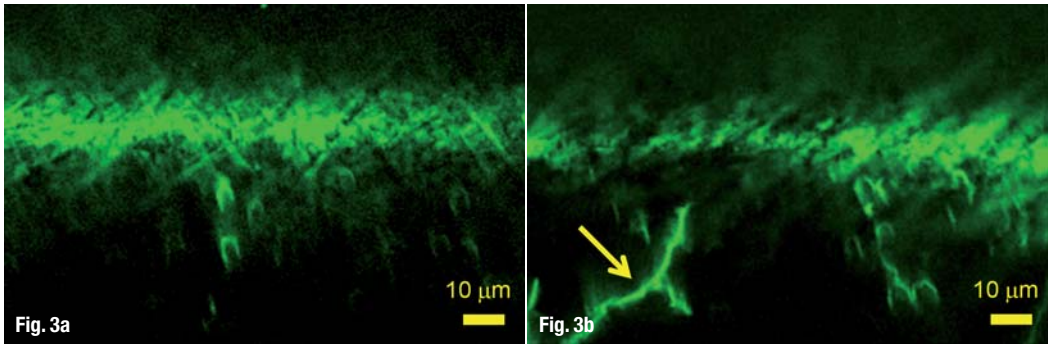


Figs. 2a & b_Experimental set up for confocal microscopy (a). Flattened surface of the incisor orientated perpendicular to the laser (b). Tooth sample immersed in distilled water.

Bleaching agents for both groups were applied for seven hours per day for 14 days. The materials were applied with a micro-brush, taking care to limit the application to the appropriate area only (Fig. 1e). Once the application of bleaching agent had been completed, the teeth were placed inside a plastic box, which acted as a moisture barrier, while keeping the bleaching agent undisturbed throughout the procedure.

After each daily application, expended bleaching material was first removed with a clean micro-brush. The area was then cleansed with water and blotted dry. Finally, the teeth were rinsed with air-water spray for 20 seconds. A cycling treatment methodology was employed. While not being actively treated, the teeth were stored in artificial saliva (Saliva Substitute, Roxane Laboratories). Up to the point of the microscopic examination, the tape covering the control group area remained in place (Fig. 1f).

Before confocal microscopic evaluation, the teeth were submerged in Texas Red dye with Dextran for 24 hours. A two-photon microscope (LSM 510 Meta, Carl Zeiss) was used to detect the fluorescence under an Argon 488 laser (Fig. 2a). Each area was examined up to a depth of 100 µm. The flattened enamel surface was orientated perpendicular to the laser beam with sticky wax and the whole tooth sample was placed under water contained in a Petri dish (Fig. 2b). Samples were viewed with a 5X/0.16 objective, focusing approximately 5 to 100 µm below the surface. Images were relayed to a computer monitor for viewing. Additional images were made



Figs. 3a & b_OP control area 6 µm subsurface (a). OP-treated 10 µm subsurface (b). Arrow indicates subsurface crack.

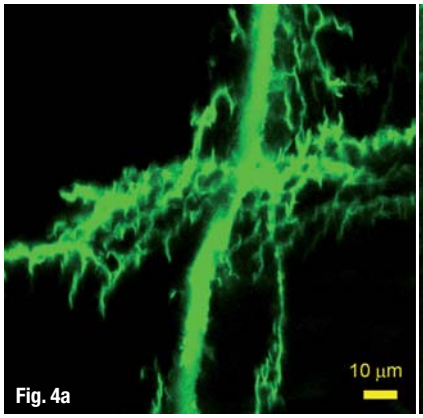


Fig. 4a

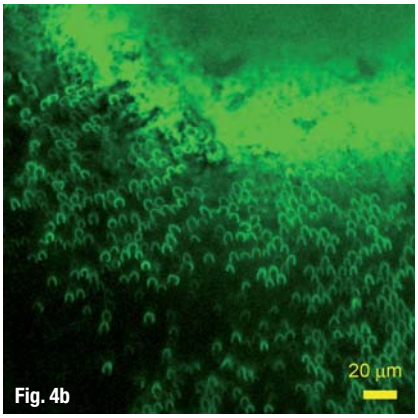


Fig. 4b

Figs. 4a & b_OP control area 28 µm subsurface (a). The dye penetrated via the crack. OP-treated 24 µm subsurface (b).

(10X/0.3 objective). High-resolution confocal microscopic images were then obtained.

_Results

Figures 3 to 6 show representative confocal micrographs of treated sections and untreated sections. Figure 3a shows 6 µm subsurface of 10 % carbamide peroxide control area and Figure 3b shows the treated 10 µm subsurface. In Figure 3b, a subsurface crack is visible. As evident from Figures 3a, 4a, 5a and 6a, there was an uptake of dyes in the control groups.

From all our observations, there were no significant subsurface porosities observed up to a depth of 100 µm, the limit of our methodology. The dye was associated mainly with enamel cracks as the depth of observation increased. The periphery of the superficial enamel prism shows increased uptake of the marker, indicating a possible route for the oxidation product to diffuse through enamel surface. General observations indicate that the dye followed inherent cracks to deep areas in the enamel subsurface (Figs. 3b, 4a, 6a & b).

_Discussion

Figs. 5a & b_ACP control area 12 µm subsurface (a). ACP-treated 48 µm subsurface (b).

While most studies have evaluated the effect of whitening on the morphological effect of enamel and dentine, the present study focused on the significance of subsurface enamel pores and defects. In our study, we did not find any significant subsurface porosities, observed up to a depth of 100 µm. The cycling treatment methodology with artificial saliva storage for 17 hours may have repaired some of the initial damage done by the bleaching. We also did not find any difference between the ACP and the OP groups. Opalescence PF 10 % contains fluoride and potassium nitrate, but it is unknown whether these two ingredients perform the same desensitising function as claimed by ACP.

Iwamoto *et al.* showed similar negative results when silver nitrate was used as the staining agent. In that study, no penetration was seen in the enamel of any of the groups.[18] However, we did see penetration of the dye in the periphery of the enamel prism in both the control and the treatment groups for both whitening materials. The increased uptake may be due to the removal of the organic components from the superficial enamel layers by the bleaching agent. The uptake of dyes in the control groups was not expected. This may be explained by the fact that exposed enamel is under constant attack in the oral environment. The weakened pores may be the inroad for chromopores and peroxide alike.

The penetration of the dyes was especially noticeable when we followed surface cracks to a depth of 100 µm (Fig. 4a). The uptake of dyes through the cracks may be clinically significant, since it may explain why certain patients are especially sensitive to bleaching. If diffusion through the enamel intercrystalline spaces is the sole cause of sensitivity, one would expect to find higher incidence of severe sensitivity.[19] However, if subclinical defects/cracks are the cause, the clinical picture of severe sensitivity reported to be around 4 % can be better explained. Enamel cracks or lamellae have been suggested to be initiation sites for caries.[20]

As seen in Figure 4b, the periphery of the enamel prism shows increased uptake of the marker, indicating a possible route for the oxidation product to diffuse through the enamel surface. Based on our findings, we hypothesise that peroxide initially penetrated into and through the enamel intercrystalline spaces to reach the enamel dentine junction and dentine regions. Indeed, *in vitro* experiments by a number of authors have demonstrated the penetration of low levels of peroxide, from a range of peroxide products and solutions, into the pulp chambers of extracted teeth after exposure times of 15 to 30 minutes.[21–23] One would expect that diffusion of peroxide through

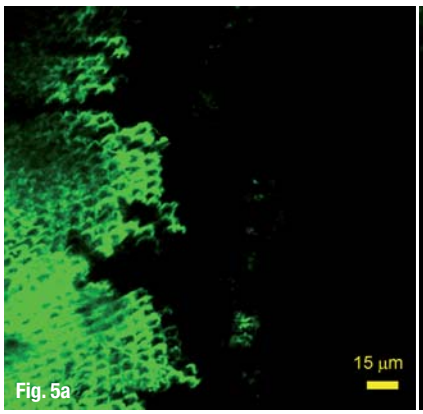


Fig. 5a

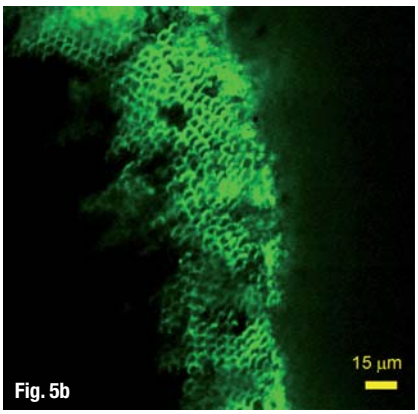


Fig. 5b

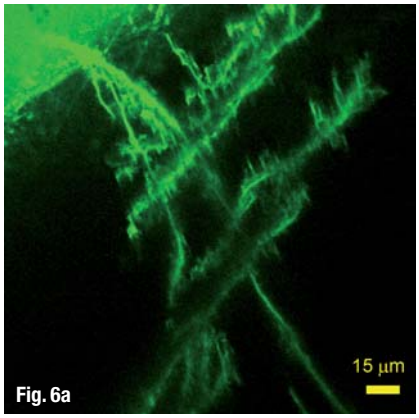
the intercrystalline spaces would be easier for peroxide, since the Dextran dye we used in our study has a high molecular weight of 3,000 to 70,000, while that of a hydroxyl radical is 17.

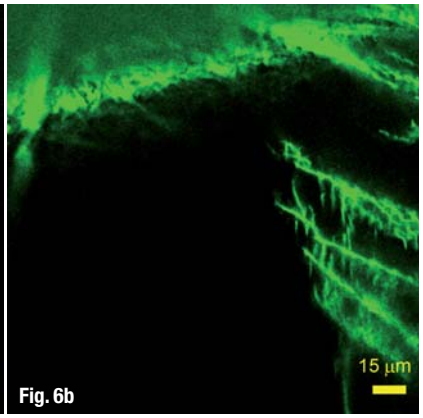
Our study did not find any evidence to support the claim that the addition of ACP reduces tooth sensitivity by decreasing the size of the pores. Another study looking at the simultaneous application of admixture solutions containing citric acid and sodium fluoride did find formation of CaF_2 globules deposited on the enamel surface. However, the CaF_2 globules deposited on the enamel surface appeared to be unable to prevent the alteration of the apatite structure during further exposure to acidic agents. No evidence of fluoride-induced recovery of the modified apatite structure was found. As for whether ACP can repair the pores simultaneously, previous studies of the repair of etched enamel reported that partial recovery from such damage takes several months *in vivo*.[24] It is unlikely that our study duration of 14 days could have offered noticeable repair.

Hypothetically, ACP could reduce the effect of sensitivity if the pore sizes were reduced. However, such reduction would also prevent efficient diffusion of hydroxyl radicals to effect bleaching. Instead, ACP could act via different mechanisms, for example by influencing the type of anion or radicals formed. Hydrogen peroxide can form a number of different active oxygen species, depending on reaction conditions, including temperature, pH, light and presence of transition metals. Whether one type of anion or radical is less prone to causing sensitivity deserves further study.

It has also been suggested that ACP may have the ability to directly depolarise nerve endings.[25] Additionally, in the generation of ACP by combining calcium nitrate and potassium phosphate, 0.25 % of potassium nitrate is generated.[26] A recent study on traditional low sensitivity whiteners raised the question of whether the low level of potassium nitrate generated as a by-product was, in fact, clinically relevant.[27]

Clinical studies have shown that ACP was effective in reducing sensitivity.[16,25] However, a recent *in vivo* study of 9 % H_2O_2 whitening strips with fifty subjects indicated that daily use of a Casein phosphopeptide-amorphous calcium phosphate paste in conjunction with tooth whitening has a minimal effect on tooth sensitivity compared with a placebo paste. Our study result raised the question of whether ACP might exert its desensitising effect through a mechanism other than decreasing the size of subsurface pores.





Figs. 6a & b_ACP control area 46 µm subsurface (a). ACP-treated 46 µm subsurface (b).

It is recognised that our study was limited by its small sample size and we only evaluated the enamel substrate. Severe sensitivity can be initiated through exposed dentine substrate. Another criticism may stem from the argument that cracks, which were shown in this study, may have been induced by extraction forces. Further studies need to be performed to explain the mechanism of ACP desensitisation better.

_Conclusion

Uptake of dye through enamel intercrystalline spaces exists in both control and bleached enamel surfaces. Whitening products with and without ACP did not appear to decrease the size of these spaces. Subclinical enamel defects/cracks may be the cause of severe bleaching sensitivity.

_Acknowledgement

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Editorial note: A complete list of references is available from the publisher.

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