

High Resolution TEM Characterisation of Hydrogen Peroxide Treated Tooth Structures

Research Article

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Abstract

Objective: Previous studies reported that bleaching agents are capable of altering the outer enamel or exposed dentine surfaces. In this study, transmission electron microscopy (TEM) characterisation in combination with energy dispersive x-ray spectroscopy (EDS) elemental mapping was performed to investigate the effect of hydrogen peroxide (HP) on the sub-surface of tooth structures.

Materials and Methods: Bovine incisors (n=6) were assigned to three groups for treatments: 30% H₂O₂ pH 3, 30% H₂O₂ pH 7 and 1M sodium hydroxide (NaOH) for 16 hours. Samples were exposed to direct treatment with agents to allow easy access of the agents and uniform treatment across the entire sample sub-surfaces. Specimens were immersed in Karnovsky's fixative for three days, embedded in epoxy and polished. Using focussed ion beam (FIB), samples were milled ~50-100 µm below the treated polished surface to feature the ultra-fine structures for TEM analysis.

Results: Enamel rods, inter-rods and crystalline structures within rods were discerned for sound and H₂O₂ treated enamel. No structural difference in the mineral dense peritubular region or fibrous protein rich inter-tubular dentine was observed after acidic or neutral H₂O₂ treatment. A complete loss in the structural integrity of enamel rod was observed after NaOH treatment for 16 hours without having any impact on dentine structure.

Conclusions: TEM analysis produced high quality sub-surface images of tooth structures and revealed no deleterious effect on the structural integrity of the sub-surface enamel or dentine after direct bleaching with hydrogen peroxide.

Introduction

In recent years, aesthetic dental treatments such as tooth whitening has gained popular demand from patients and established as a rapidly growing field of dentistry. Consumers are more conscious of their image and believe in a brighter smile as a way of maintaining beauty and health. Bleaching of teeth has a long history since 1864 and the observation of tooth lightening with Carbamide peroxide in 1989 marked the beginning of using 10% CP in a mouth guard [1].

The fundamental bleaching procedure to bleach the discoloured teeth exists in the form of dentist-supervised night guard bleaching, in-office bleaching, and OTC bleaching [2]. Various forms of OTC bleaching products are available in market in the form of gels, paint on brush, mouth rinses, chewing gums, tooth pastes, strips etc. The choice of procedure and resulting efficacy of the whitening treatment depends on many factors such as origin

or cause of tooth discoloration, type of discoloration, location and depth of stain residing tooth, affinity of the stain to enamel and dentine tissues, age and gender of patient, concentration and time of exposure to the bleaching agent.

Today many tooth whitening products are available in market with a range of active agents including hydrogen peroxide, carbamide peroxide, sodium perborate, chlorite and others. These products have been used alone or in combination with activators to increase the efficacy of whitening.

Hydrogen peroxide is the most commonly used bleaching agent of this era [3]. It decomposes and produces high oxidising potential free radicals, perhydroxyl anions, which affect pigment macromolecules and resin matrices [4]. Although enamel is a hard and dense tissue, its highly porous structure enables hydrogen peroxide to penetrate this layer by diffusion [5]. Jiang et al. noted that hydrogen peroxide attacks the mineral and organic matrix of the dentine layer, and hypothesize that the organic components

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are removed, and the mineral components collapse and form a layer to protect the underlying dentine layers from any further attack [6].

To date, most of the studies reported on mineral content, surface morphology, surface and sub-surface micro hardness and other mechanical properties associated with hydrogen peroxide treatment utilised techniques such as scanning electron microscopy, atomic force microscopy, confocal laser microscopy, attenuated total reflection-Fourier transform infrared spectroscopy and Raman spectroscopy [7-17]. The combination of using focussed ion beam transmission electron microscopy (FIB-TEM) for studying the mineralized dentine has been reported over the years [18, 19]. To our knowledge limited information is available on the use of high resolution (TEM) for examining the sub surface microstructure of enamel and dentine after hydrogen peroxide treatment.

Compared to US, tooth whitening in Europe is not very popular and this might be because of the lingering concerns about the safety associated with whitening procedures [2]. Several studies have been conducted to evaluate the safety of using tooth whitening products based on carbamide peroxide and hydrogen peroxide. It has been suggested that considering the dosage and application mode of tooth bleaching, the exposure to the material during the bleaching procedure is inadequate to cause acute systemic toxicity [20].

A review on the safety of using hydrogen peroxide based materials confirmed that there is no evidence of significant health risks associated with tooth whitening and any such potential adverse effects occur with the in-appropriate usage of whitening products for tooth whitening [21]. It was also reported that the peroxide containing products and solutions have no significant deleterious effect on tooth surface morphology, chemistry, surface and sub-surface micro hardness or ultra-structure. In contrast, the studies that showed an effect of peroxide have limitations in their method of not considering the *in vivo* situations of saliva remineralisation or using products that have acidic pH [22]. Considering the disparities relating to the safety of hydrogen peroxide, a well-controlled fundamental study is essential to gain insights into the impact of peroxide on the ultra-fine structure of teeth.

The aim of this study is to investigate the effect of hydrogen on teeth down to nanometre level using high resolution transmission electron microscopy to evaluate the safety of performing peroxide based tooth whitening procedures and any associated risks. In a previous study reported by Eimar et al. (2012), sodium hydroxide was used as a deproteinizing agent for studying the mechanism of whitening agents on teeth. Therefore, the effect of hydrogen peroxide on the organic content of tooth was tested by comparing the samples treated with deproteinizing solution of sodium hydroxide. EDS was used to perform elemental analysis of enamel and dentine on controls and treated sample with agents.

Materials and Methods

Specimen preparation

The study used non carious BSE certified bovine fragments of dimensions 10 x 10 mm without any cracks or defects. The

hydrated teeth samples were sectioned (low speed saw, Isomet) from the crown to the root at a distance of 1-1.5mm from the labial aspect of tooth. Each tooth was cut into two equal parts to serve samples for treatment and internal control. Samples were polished, etched with 10% citric acid for 30 seconds and cleaned in ultrasound bath. The untreated control samples for each treatment were stored in PBS at 37°C for 16h.

Chemical solutions for treatment

The following solutions were prepared for treating the samples with enamel and dentine.

- I. 30% hydrogen peroxide solution (HP) pH 3: sample was immersed in 30% w/w HP solution, (Sigma Aldrich, UK) for 16h at 37°C.
- II. 30% hydrogen peroxide solution pH 7: pH of the 30% HP solution was adjusted to 7 using 1M sodium hydroxide (NaOH) and the sample was immersed in the solution for 16h at 37°C.
- III. Deproteinizing solution: 1M NaOH solution (Sigma Aldrich, UK) was prepared and the sample was immersed in the solution for 16h at 37°C.

Dental sample preparation for TEM examination

For examination in TEM, damage-free electron transparent lamellae of 50-100 nm thickness were obtained from a two-step routine procedure.

Mechanical polishing

Samples were embedded in an electron microscopy compatible epoxy (Struers Epofix Resin) and mechanically polished (using a Struers RotoPol 21 mill) until ~50-100 µm below the original polished surface to avoid surface effects and sample preparation artifacts. This was followed by a second embedding step to fill in small voids like tubules and a subsequent ~250nm polishing step. As the samples were exposed uniformly to treatments via direct bleaching method, the locations for the TEM samples could be chosen randomly from the polished tooth surfaces.

Focused Ion Beam milling

TEM cross-sections of samples were prepared in a FEI Nova 200 Nanolab Small Dual Beam (SDB), equipped with a FIB and a field emission scanning electron microscope (SEM) column. To avoid charging and prevent ion-milling damage, C and Pt based protection layers were deposited both *ex-* and *in-situ*. The FIB lift-out method was used to retrieve TEM samples. Ga ions (from a Ga Liquid Metal Ion Source) were used to mill away excess material. Different energy milling steps ranging between 20nA and 100pA performed at 30 kV were used to obtain thin TEM-lamella. As a final cleaning step, a 5 kV milling step was performed. Instead of using a cryo-stage, current densities were reduced by a factor 4 to 6 and dwell times were shortened by a factor 18 to obtain FIB-artefact free samples. The preparation method was similar for enamel and dentine samples. As unfilled tubules would have led to re-deposition and curtaining artefacts during milling, extra care was taken during epoxy filling of tubules. Final TEM lamellae were created with dimensions of 15-20 µm width x 5 µm height and 50-100 nm thickness. The time between extraction from the

SDB and insertion into the TEM was kept below 3 minutes to minimize ambient influences.

Microscopic and elemental analysis

TEM studies were performed using a JEOL ARM 200 CFEG (Cold FEG) TEM equipped with a windowless 100mm² silicon drift detector; energy dispersive x-ray spectroscopy (SDD EDS) detector. Imaging was performed in bright field and high angle annular dark field-scanning TEM (HAADF-STEM) mode. TEM was operated at 80kV during all examinations to minimize electron beam damage.

In bright field TEM mode both elemental and diffraction contrast contributed to the image. Heavy material appeared dark and vice versa, whereas crystalline material like enamel yielded different grey values depending on the material's crystal orientation.

The HAADF-detector detected electrons that are elastically scattered after interaction with the TEM-sample, yielding mass sensitive contrast; higher brightness in the image corresponded to the presence of (larger concentration of) heavier atoms.

Two-dimensional elemental mappings were acquired in HAADF-STEM mode in combination with the EDS detector using JEOL Analysis Station software. In scanning TEM, a small electron beam diameter (~0.1 nm) in combination with the EDS detector enabled elemental analysis on the (sub) nano meter scale. In the collected EDS spectral mapping the detected signal was plotted as a function of the (element characteristic) X-ray energy.

Results

Effect of treatments on enamel

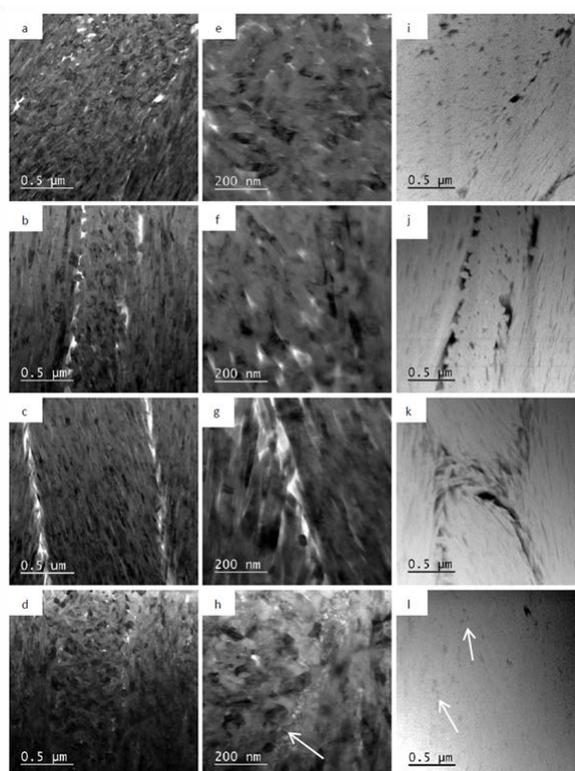
Typical detailing of the ultra-structure of sound and 16 hours treated enamel samples were examined under transmission electron microscopy and are shown in Figure 1.

The structural analysis of sound enamel demonstrated rod and interrod structures (Figure 1a, i). The rods and interrods with numerous densely packed and well organised crystalline facets were clearly visible in the bright field image at higher magnification (Figure 1e). The HAADF image (Figure 1i) showed pores at the prism-junction as dark holes within the structure.

As observed in reference sample, the overview images of 30% HP (pH 3 and 7) treated enamel depicted the crystalline structure of rods and interrods (Figure 1b-c, 1f-g). In the TEM lamella, the rod structure could be differentiated clearly from the interrod structure with the difference in the orientation of crystals (Figure 1b). The interfaces between the rods displayed voids as the untreated sample. However, a wider region of rod sheath was observed for 30% HP treated samples (Figure 1b-c and 1j-k).

The characteristic crystalline structures within prisms were visible on enamel treated with NaOH (Figure 1d). However, a significant change in the overall structure of sample was observed with the absence of structured rods and well defined boundaries between rods and interrods. The crystals were merged and no regular spacing between the facets was visible. In the detailed HAADF image (Figure 1l), the characteristic voids representing the pores between the rods are absent. In Figure 1h, porous material was

Figure 1. Images of enamel in TEM mode (a-h) and HAADF mode (i-l). Electron micrographs of sound enamel (a, e, i); treated with 30% HP solution, pH 3 (b, f, j); treated with 30% HP solution, pH 7 (c, g, k) and treated with NaOH (d, h, l).



visible at many of the prisms borders compared to other samples (denoted by white arrow). Using EDS, this porous material contain fluoride.

Effect of treatments on dentine

The electron micrographs obtained from the dentine cross-sections of control and treated tooth samples in bright field TEM and HAADF-mode are shown here.

The examination of micrographs sound dentine at higher magnifications on TEM and HAADF mode revealed a highly dense peritubular (PT) region surrounding the tubule. In TEM, the PT is characterised as a dark region (Figure 2a) whereas it appeared as a bright region in HAADF image (Figure 2i). The dark speckles appeared in TEM of PT were not visible as bright speckles in HAADF, indicated that the speckles were not correlated to denser material but to the presence of crystals. In HAADF, small voids which are intrinsic to the tooth material were visible in the PT region. The intertubular region displayed a very open fibre structure and often these regions contain far less or no material. Collagen fibres are found in the intertubular regions, characterised by the periodic banding in contrast in both modes (Figure 2e, i).

The electron micrograph of dentine treated with 30% hydrogen peroxide solutions for 16h showed the presence of PT region that was asymmetric in positioning around the tubule and inhomogeneous in thickness (Figure 2b-c, j-k). Differences and inhomogeneities in the thickness of the peritubular region were observed for control and treated samples and hence the thickness of PT region cannot be used as a measurement from the impact of treatment (Figure 2i-l). As observed in the control sample,

collagen fibres were exposed, represented by the periodic banding (Figure 2f) and crystals were visible as small dark speckles in the denser material (Figure 2g).

The NaOH treated sample displayed the denser PT region and collagen fibres in the intertubular region (Figure 2d, h and l). The tubules were not filled with resin for this sample and this could be easily distinguished from other images. It appeared that the dental material was deposited into tubules during early stages of preparation. The dark dots in the denser material around the tubule are small crystals. HAADF image (Figure 2l) showed the denser material around the tubule. The image also showed small voids in the denser material of the sample similar to other samples.

Energy Dispersive X-ray Spectroscopy (EDS) mapping

The HAADF-STEM images shown in Figures 1 and 2 indicate the areas that were scanned during EDS acquisition.

A representative EDS spectral mapping acquired from untreated dentine is shown in Figure 3. Apart from the major constituent elements such as Ca and P, minor amounts of N (6 atom %), F (about 1 atom %) and Mg, Na (< 0.5 atom %) were detected in the regions analysed.

Figure 4 shows the two-dimensional EDS map of carbon in the enamel as a function of treatment. Although C was detected in between the rods or rod sheath of untreated teeth, the signal was not acquired in hydrogen peroxide and sodium hydroxide treated enamel samples. The C content can be linked to the presence of protein material. It was reported earlier that HP and NaOH attacks proteins, which are C-based material [34] and this could explain the absence of C in treated sample.

Figure 2. Electron micrograph of dentine in TEM and HAADF modes. Electron micrographs of sound dentine (a, e, i); treated with 30% HP solution, pH 3 (b, f, j); treated with 30% HP solution, pH 7 (c, g, k) and treated with NaOH (d, h, l).

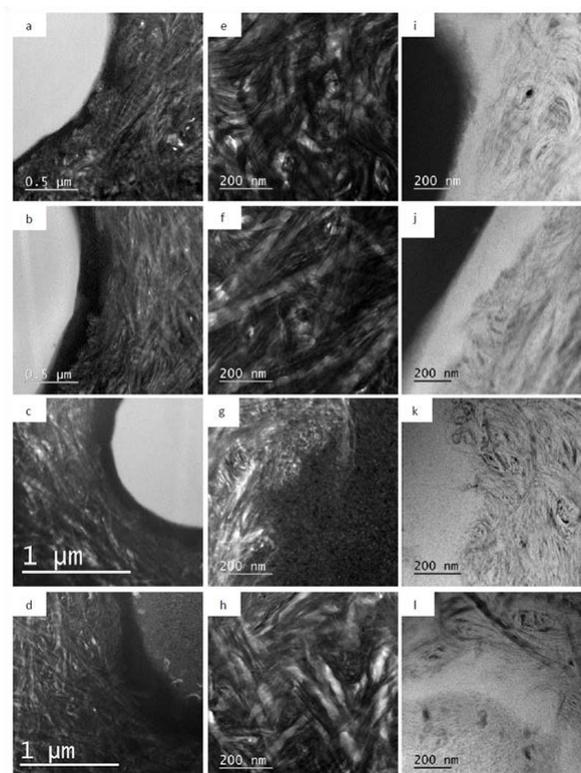


Figure 3. Raw counts EDS mappings (intensities represent counts) of untreated dentine sample. 256*256 pixels resolution (3x3L filter), 0.1 msec/pixel acquisition time, summation over 219 frames.

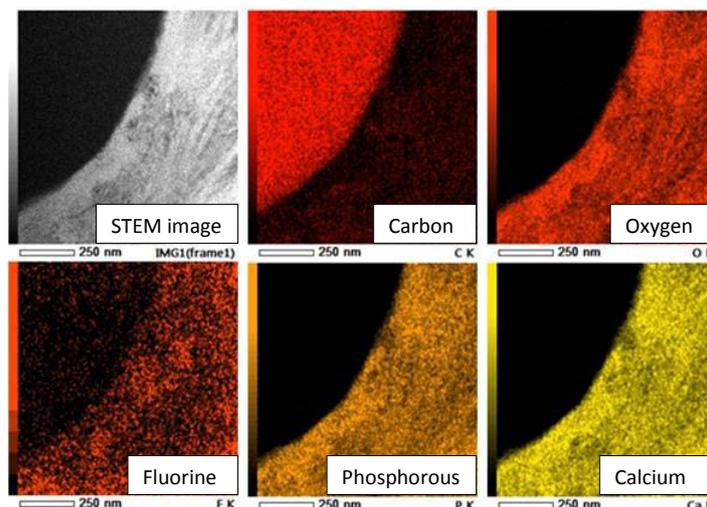
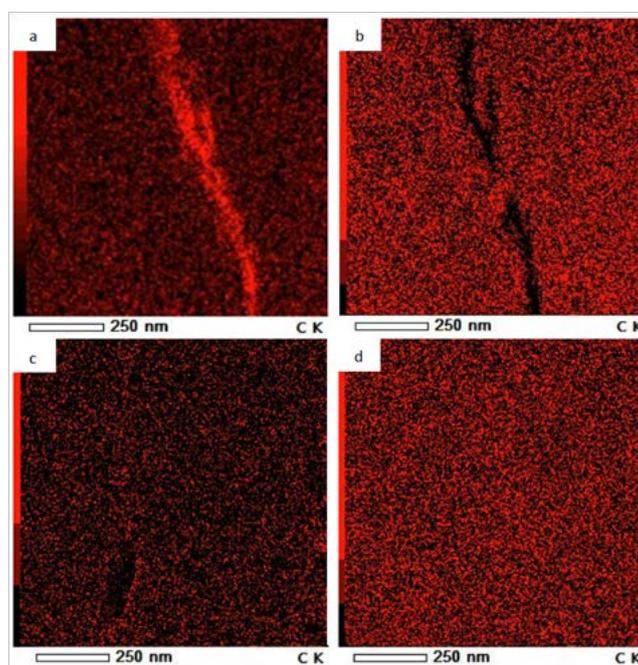


Figure 4. EDS mapping of carbon in (a) untreated (b) 30% H₂O₂, pH 7 (c) 30% H₂O₂, pH 3 (d) sodium hydroxide treated enamel samples.



The entire set of EDS mappings of enamel and dentine samples treated with HP and NaOH can be found in the [supplementary material](#).

Discussion

Dentine is a complex hydrated biological composite structure for which only limited structure-property relationships are available [23]. An increase in the demand for tooth whitening led to the release of enormous number of products in the market. Therefore a better understanding of the ultrastructure of dentin after whitening treatments is essential to provide valuable insights into the safety of using whitening products.

In this study, we investigated the effect of hydrogen peroxide and sodium hydroxide on enamel and dentine using a direct bleaching method to explore any underlying deleterious effect

from treatments. Acidic and neutral hydrogen peroxide solutions were tested to understand the effect of pH of whitening products and sodium hydroxide as an agent to analyse the role of proteins in the overall tooth structural integrity. FIB produced thin lamellae of mineralised teeth samples with minimal damage to examine the morphological changes after 30% HP treatment and deproteinization.

Bovine enamel and dentine were selected for this feasibility study as the crystalline orientations of bovine enamel matches to human, has more uniform composition and is more susceptible to acid attack due to variations in the hydroxyapatite lattice [24, 25]. Moreover, bovine enamel is more porous and demineralises faster than human enamel [26]. Therefore with bovine teeth, any structural changes that eventuate from hydrogen peroxide or sodium hydroxide treatments could be clearly and rapidly captured under high resolution microscopy. The use of bovine teeth also enables comparison with several other studies, which

have reported their use in adhesion studies, caries-like lesions, micro hardness and lesion depth studies as an alternate substrate to human teeth [26-30].

TEM examination of 30% hydrogen peroxide treated sample revealed no difference in the structural integrity of enamel rods or fibrous structure of dentine at ~50-100µm depth. This is in agreement with many previous studies reported on the minimum or insignificant effect of acidic and alkaline 30% hydrogen peroxide solution [14, 31-34]. However, studies conducted by Sun et al. (2011) observed less deleterious effects of neutral 30% HP on enamel than acidic solution [10]. Moreover, several other studies have reported a decrease in the microhardness of dentin following 30% HP solution at pH 3 and reduced *in vitro* fracture resistance of dentin after prolonged use of leaching products [35, 36]. As reported by Chng et al., the surface alteration on intertubular dentine resulted from hydrogen peroxide treatment occurred predominantly from its oxidising property rather than the acidic pH [37]. The observation is similar to our study where no alteration in the ultra-structure of dentine was visualized with acidic or neutral hydrogen peroxide solutions.

The wide difference between the studies could be derived from the preferred *in vitro* method or technique used, type of tooth studied, source of peroxide, concentration of peroxide and time of exposure, effect of other non-active ingredients in formulation or presence of saliva as a potential remineralising agent etcetera [22]. In order to avoid the complexity derive from the presence of components in saliva, the immersion of tooth samples in artificial saliva before or after treatments was excluded from the protocol of this current study.

In contrast to HP treated teeth, enamel treated with sodium hydroxide revealed a complete loss of the defined structure of enamel rods. This might be due to the fact that deproteinisation of enamel resulted in the destruction of highly organized array of very fine hydroxyapatite crystals embedded in protein rich rod sheaths and within the rods themselves [38]. Proteins which constitute only a small fraction of enamel disperse through enamel and bind together the hydroxyapatite crystals within the rods and the rod sheath [39].

High resolution imaging of the dentine revealed the presence of highly dense peritubular regions (HAADF mode), needle like crystallite structures and characteristic periodic banding of protein matrix featuring the collagen fibril structures. TEM analysis of treated dentine did not show any evidence of the destruction of peritubular region or fibres within the intertubular region. The microstructure of enamel and dentine observed to be similar between tooth structures treated with acidic and neutral hydrogen peroxide. This might have been a result of the examination of samples ~100µm below the original exposed surface. Therefore the final TEM lamella might not have captured any superficial morphology change resulted from acidic peroxide.

With EDS mapping, the presence of Ca, P, O, F and traces of Na, Mg and Cl were detected in the enamel of control and treated samples. Carbon was only present in the rod sheath area of control enamel with the absence of nitrogen in the enamel of both control and treated samples. We postulate that the absence of carbon in treated samples could be due to the removal of extrinsic hydrocarbons such as stains. The presence of porous

material was observed in sodium hydroxide treated sample at several prism borders unlike other samples and using EDS, this porous material appeared to contain F. EDS analysis of dentine specimens showed the presence of C and N, representing the existence of organic contents such as proteins. However, no effects of treatment were found in the EDS data of the dentine samples.

The safety of using tooth whitening products and risks associated with the treatment procedure is still unclear and controversial. Experts in this field recommended the involvement of dental professionals in bleaching treatments to reduce the risk that might occur during the treatment and for correct diagnosis of the problem which resulted in tooth discolouration [20]. We demonstrated (at least to first order) that the ingestion of hydrogen peroxide whitening agents into the complex hard tissues of the teeth does not cause significant damage to the mineral and protein constituents. Although the sub-surfaces of the enamel and dentine are not affected by HP treatment, it is possible that some alteration were occurred on tooth surfaces, which might not be clinically significant. The normal structural morphology could be restored with the natural remineralisation of saliva and with the practice of following a suitable post treatment care under the supervision of a professional dentist. However, further studies using extracted human teeth in an ideal oral environment and subsequent clinical studies are required to confirm such suggestions and for accurately predicting the effect of whitening agents on teeth.

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Special Issue on

"Tooth Bleaching Agents"

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