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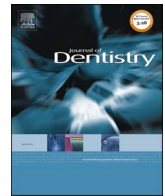
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The effect of Hydrogen Peroxide treatments on dental enamel porosity and protein structure and its long-term implications on tooth hardness and optical properties

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ABSTRACT

Objective: The aim of this study is to investigate how hydrogen peroxide (HP) treatments affect enamel porosity and protein structure, as well as their impact on tooth shade and hardness over time.

Methods: Fifty healthy teeth were collected from adult patients. Teeth were randomly divided into 2 groups, the first was incubated with 30 % HP while the second, the control group, was incubated with distilled water (DW). Tooth shade and enamel microhardness were evaluated using a digital spectrophotometer and a Vickers tester, respectively, at different time intervals after treatment. The specific surface area of the enamel was measured using a surface area analyzer and the Brunauer-Emmett-Teller (BET) equation. Protein structure was analyzed using circular dichroism (CD) spectroscopy and dynamic light scattering (DLS).

Results: Shade analysis revealed that, in the short term, HP treatment significantly increased lightness and Hue and decreased chroma compared to DW ($p < 0.05$); however, 1 week after treatment some of the initial gains in tooth lightness were partially lost. Hardness analysis revealed significant decreases in microhardness in the bleached group compared to the control group ($p < 0.05$). BET analysis revealed that HP treatment increased enamel surface area and reduced its average pore size. CD and DLS analyses showed that proteins from HP-treated teeth mostly adopted a non-random conformation and had smaller average protein sizes compared to the control.

Conclusion: HP treatment lightened tooth shade and significantly reduced enamel microhardness over time. This could be related to changes in surface area and porosity caused by denaturalization of the enamel proteins.

Clinical significance: Our findings showed that HP induced denaturation of enamel proteins, resulting in increased enamel porosity and reduced microhardness. These changes in enamel properties could help explain clinical complications observed with these treatments such as increased sensitivity.

1. Introduction

Tooth shade plays a crucial role in boosting self-confidence, enhancing interpersonal communication, and reducing psychoemotional concerns about being perceived as unattractive [1]. In recent decades, aesthetic dentistry has advanced significantly, with a marked increase in the popularity of certain procedures, especially tooth whitening. This treatment has become a key aspect of personal care, enhancing both the appearance of patients' teeth and the overall

patients' quality of life [2]. While dental bleaching continues to be a popular cosmetic treatment, there are growing concerns about the long-term effects of bleaching agents on dental health. As a result, researchers and dental professionals have increasingly focused on examining the impact of commonly used bleaching agents, such as hydrogen peroxide (HP) and carbamide peroxide (CP), on dental structures [3–6].

HP acts as a strong oxidizing agent by producing free radicals, reactive oxygen molecules, and HP anions [7]. Although several hypotheses have been proposed to explain HP's bleaching effect [8], the

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most widely accepted theory suggests that HP whitens teeth by oxidizing the organic matrices within the enamel, without altering its relative organic or inorganic content [4]. This oxidative process results in tooth whitening by reducing the relative translucency of enamel [5,9]. A more recent study has suggested that HP may also whiten dentin by oxidizing aromatic amino acids in dentin phosphoprotein, thereby resulting to a lighter tooth shade [5]. However, the effect of this oxidation on the enamel protein structure remains underexplored.

Dental enamel is a highly mineralized, hierarchical composite material composed of a dense mineral phase (approximately 96 % by weight, 90 % by volume), a soft organic phase (around 1 % by weight, 2 % by volume), and water (about 3 % by weight, 8 % by volume) [10]. It is primarily composed of hydroxyapatite crystals organized within a protein matrix, mainly composed of enamelin and amelogenin, which provide essential structural support to the enamel rods [10]. Although proteins constitute <1 % wt of enamel, they are crucial for maintaining enamel's mechanical properties and stability [11]. Despite their small proportion, several studies have shown that these proteins significantly influence the fracture resistance of enamel accommodating mechanical stresses, with deproteinized enamel exhibiting lower fracture toughness [12,13].

Despite HP's effectiveness in teeth whitening, its impact on the properties and integrity of the dental enamel remains subject of debate. While some studies reported no significant changes in enamel properties [14–16], others suggested that bleaching agents can affect both the surface and bulk properties of enamel [17–21]. Sulieman et al. [14] found that 35 % HP itself had no harmful effects on enamel or dentin, but the pH of the bleaching materials might cause adverse effects during the procedure. Additionally, Faraoni-Romano et al. [16] reported no changes in microhardness or surface roughness of bovine enamel after bleaching with low (10 % CP) and relatively high (38 % HP) concentrations of bleaching agents. In contrast, several studies on the mechanical properties of enamel have demonstrated that HP notably reduced the enamel's hardness and modulus of elasticity [17,18,22], as well as the fracture toughness and wear resistance [3,12,13]. Additionally, an increase in surface roughness and erosion has been reported as determined by scanning electron microscopy and laser profilometry [23,24]. However, there is a lack of studies investigating the long-term effects of HP on both the shade of teeth and the hardness of enamel over time. Given the widespread use of HP in whitening treatments, this limitation in the existing research highlights the need for further investigation into these long-term implications.

While most research has focused on changes in the mechanical properties of enamel, only a few studies have explored the impact of bleaching agents on the organic components of enamel. Understanding these chemical changes is crucial for a thorough evaluation of the long-term effects of whitening treatments. In this regard, techniques such as Circular Dichroism (CD) and Dynamic Light Scattering (DLS) are very useful for characterizing the structures of proteins in dental enamel and dentin [25,26]. CD is a spectroscopic method that reveals the secondary structure of proteins, including their folding, conformation, and stability. DLS, on the other hand, analyzes protein size distribution and aggregation, providing insights into changes in size and morphology [27]. These techniques, which as of now have never been used to investigate how HP may affect enamel proteins, could provide unique insight into the molecular mechanisms involved in tooth bleaching and help guide product development and clinical decision-making for better treatment outcomes.

Therefore, the objective of this study is to examine the effects of HP on the shade and microhardness of enamel over time, and assess changes in the enamel surface morphology and the structure of enamel proteins. Collectively, these methods offer a comprehensive approach, thereby deepening our understanding of the effects of widely used bleaching treatments on enamel. We hypothesize that HP tooth whitening treatments denaturalize tooth enamel proteins, subsequently affecting enamel porosity and mechanical properties.

2. Material and methods

2.1. Sample preparation

This study was approved by the McGill University Health Centre Ethical Committee (IRB No A01-E02-188) and Hamad Medical Corporation (MRC-02-22-469). After obtaining informed written consent from the patients, 50 sound teeth (upper anterior teeth and premolars), freshly extracted for periodontal and orthodontic reasons, were included in the study. The teeth were free of caries, demineralization, fractures, restorations, or prior bleaching treatments. Upon extraction, they were preserved in a 10 % formalin solution (BF-FORM, Fisher Scientific, Montreal, Canada) for one week [28]. The teeth were then cleaned of soft tissue remnants in an ultrasonic bath (FS20D Ultrasonic, Fisher Scientific, Montreal, Canada) with deionized distilled water for 60 min at 25 °C. Afterwards, the teeth underwent prophylaxis for one minute with a low-speed dental handpiece (M5Pa, KAB-Dental, Sterling Heights, MI, USA) using silicon carbide cups (Pro-Cup, SDS Kerr, Orange, CA, USA) and low-abrasive dental prophylaxis pumice (CPR™, ICCARE, Irvine, CA, USA). The teeth selected for shade and microhardness analysis were rinsed in an ultrasonic bath with deionized distilled water and stored for further analysis in labeled Eppendorf tubes containing 10 % formalin solution. The entire experiment is presented in Fig. 1.

2.2. Tooth color measurements

The sample size calculation for the color measurements was conducted using G*Power v3.1.9.4. With a study power of 80 %, an alpha error probability of 0.05, and an effect size of 0.3 [29], the calculation considered two groups and five time points. Under these conditions, the minimum required sample size was 16 in total. Accordingly, for color analysis, 20 teeth were randomly allocated into two groups ($n = 10$): Group 1 (bleached group), where the teeth were treated by incubation with 30 % HP (Sigma-Aldrich, Darmstadt, Germany), and Group 2 (negative control), where the teeth were not subjected to bleaching but were kept in distilled water (DW). Bleaching was conducted in one session with applications lasting 30 min. Following the procedure, the teeth were rinsed under tap water to remove the bleaching agent and then stored in DW.

Shade was assessed using a digital spectrophotometer (Vita Easyshade, Vita Zahnfabrik, Bad Sackingen, Germany) [20,30]. To prevent dehydration, which can cause clinically significant shade changes [31, 32], all teeth were kept moist throughout the measurement process. The color of each tooth was analyzed using the Munsell color system (L^* , C^* , and H^*), as it is the basis for most tooth shade guides used in dental practice [4]. The Munsell scale system was selected due to its ability to correlate to the chemical composition of materials [4,33]. Measurements for each parameter were recorded in the middle third of the tooth specimens at baseline, and then at 14 h, 1 week, 2 weeks, 3 weeks, and 4 weeks post-bleaching. Shade measurements were performed in triplicate for each tooth, and the mean and standard deviations of these measurements were calculated.

2.3. Microhardness measurements

The sample size calculation for the microhardness measurements was conducted using G*Power v3.1.9.4. The calculation set at a power of 80 %, an alpha error probability of 0.05, and an effect size of 0.4 [34] indicated that for a repeated-measures ANOVA design for two matched groups, assessed at five time points, the minimum required sample size was determined to be 5 per group. For hardness evaluation, the crowns of the teeth were separated from the roots at the cemento-enamel junction, and were sectioned mesiodistally into two equal halves using a carbide bur (FG56, SDS, Kerr, Orange, CA, USA) mounted on a high-speed handpiece (TA-98LW, Synea, Bürmoos, Austria). This

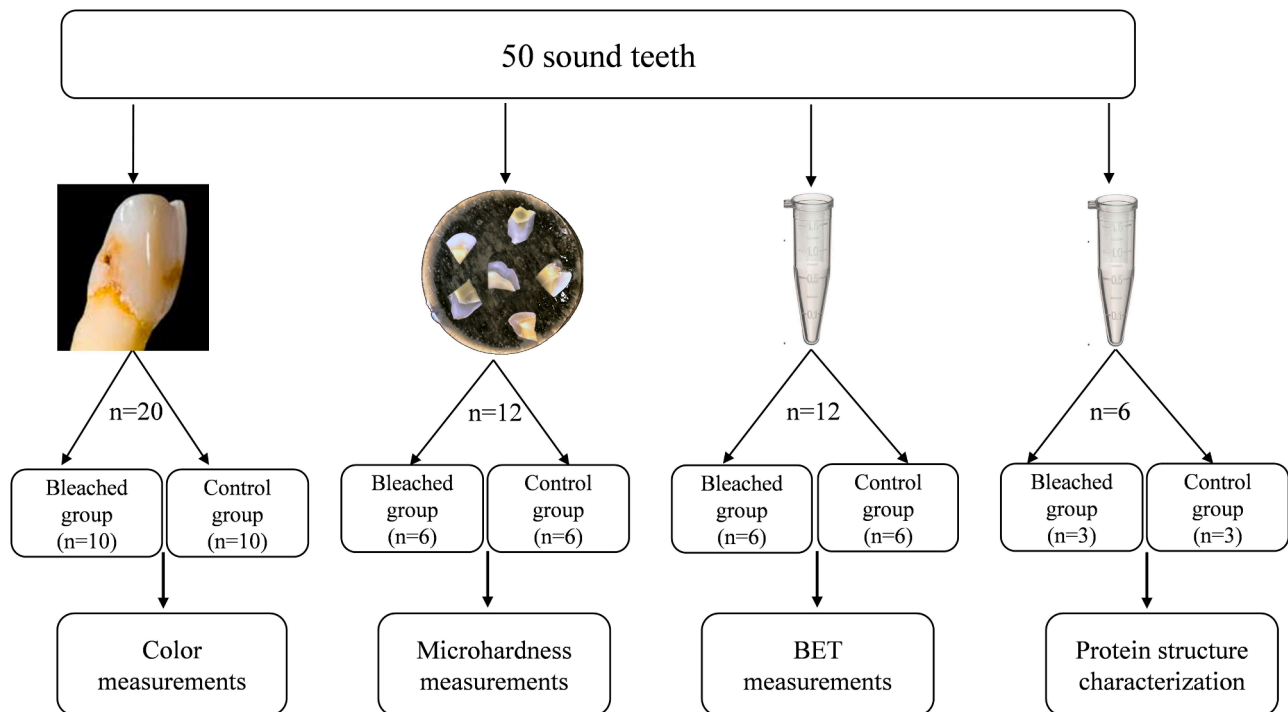


Fig. 1. Diagram describing the methodology of the study.

sectioning was performed under a continuous flow of deionized distilled water to prevent thermal damage. The paired segments collected from each tooth were divided into 2 matching groups ($n = 6$), each consisting of 6 matching tooth segments. The segments in each group were then embedded in clear methylmethacrylate resin (DP-Ortho-F, DenPlus, Montreal, Canada). The resin blocks were carefully mirror polished using progressively finer grades of silicon carbide paper (Paper-c wt, AA Abrasives, Philadelphia, PA, USA) (240, 400, 600, 800, and 1200 grit) and finished with a polishing cloth and colloidal silica (smooth). The blocks were then randomly assigned to two treatment groups: Group 1, the bleached group, received treatment with 30 % HP (Sigma-Aldrich, Darmstadt, Germany) as described above, while Group 2, the negative control group, was exposed to DW.

Microhardness was measured by applying a 100 g force for 10 s to the resin-embedded enamel specimens using a Vickers microhardness tester (Clarck CM100AT, HT-CM-95,605, Shawnee Mission, KS), with the assistance of computer software (Vision PE 3.5, Clemex Technologies Inc., Shawnee Mission, KS), based on images captured by a built-in camera. To account for variability within the enamel layer, ten readings were taken from each specimen at each time point. The measurements were made halfway between the dentin-enamel junction at the enamel surface. A minimum distance of 50 μm between readings was maintained. The mean microhardness values were calculated at baseline (before treatment) and reassessed at 14 h, 1 week, 2 weeks, 3 weeks, and 4 weeks following the application of the bleaching material.

2.4. BET gas adsorption measurement

To evaluate the enamel surface area and pore size after exposure to HP and DW, the Brunauer-Emmett-Teller (BET) gas adsorption method was utilized [35]. Enamel powder was obtained by grounding enamel sections from 12 teeth using a carbide bur (FG56, SDS Kerr, Orange, CA) attached to a high-speed dental handpiece (TA-98LW, Synea, Bürmoos, Austria). The ground enamel was rinsed with deionized distilled water, and after the water evaporated, the enamel powder was freeze-dried using a freeze-dryer (Virtis, SP Scientific, Warminster, Pennsylvania, USA). Following this, the enamel powder was homogenized using a

Vortex mixer (Benchmark Scientific, USA), weighed on a scale, and divided into six test tubes. Three powder samples were randomly selected for treatment with HP, and the remaining 3 were allocated as control samples. The HP treatment was carried out by suspending the enamel powder samples in 1 ml of HP (30 %) solution (Sigma-Aldrich, Darmstadt, Germany) for 5 min. Afterwards, the suspension was washed with 10 ml of distilled water, centrifuged (2000 rpm for 2 min, EBA 21 Hettich Zentrifugen, Tuttlingen, Germany) and rinsed 3 times. The supernatant was discarded and the powder was freeze-dried using a freeze-dryer (Virtis, SP Scientific, Warminster, Pennsylvania, USA). Prepared samples were analyzed using a surface area analyzer (ASAP 2420, Micromeritics) at 77K. The samples were first dried at 90 °C for 30 min, then degassed at 150 °C for 4 h prior to analysis. They were then placed in a sample tube and heated under vacuum to eliminate contaminants and water from their surfaces. The sample tube was then inserted into the analysis port of the 2420 Accelerated Surface Area and Porosimetry System (Micromeritics, Norcross GA, USA) for automated analysis. Specific surface area and pore size were calculated for each group following the standard BET method.

2.5. Protein structure characterization

CD spectroscopy was employed to assess the effect of HP treatment on the secondary structure of proteins in enamel in comparison with DW. DLS was used to assess changes in the size distribution of enamel proteins following exposure to HP and DW [25–27]. For protein analysis experiments, freshly extracted teeth were decontaminated in 70 % ethanol for 1 hour [36], rehydrated in DW, and then stored at –20 °C to avoid protein damage during tooth storage or fixation. Six teeth were randomly allocated to two groups for treatment with either HP or DW as described above. After treatment, enamel segments were separated from the dental crowns using a diamond bur cooled with distilled water. The enamel sections were then submitted for protein extraction. First, the tooth enamel segments were ground with a pestle and mortar in liquid nitrogen until a fine powder was obtained. Enamel powder from both bleached specimens and those exposed to DW was demineralized in HCl as described elsewhere [37]. Briefly, the specimens were placed in a 10

% HCl solution at 4 °C until they were fully dissolved. The solution was then centrifuged at 10,000 rpm, and the supernatants were dialyzed against HEPES buffer (pH 7.4) before being submitted for analysis by CD and DLS [25,27].

CD spectra were recorded using a J-815 spectropolarimeter (Jasco, Easton, MD, USA) to measure the absorbance difference between left and right circularly polarized light. The resulting data, which displayed elliptical polarization, was used to monitor the secondary structure in the far UV region between 190 nm and 250 nm.

DLS analysis was conducted using a ProteinSolutions DynaPro-E instrument (Wyatt Technology, Corp., Santa Barbara, CA, USA), with data acquisition managed by Dynamics (Version 6) software (Wyatt Technology Corp).

2.6. Statistical analysis

Data analysis was performed using SPSS software, version 20 (IBM Corp., Armonk, NY, USA). Descriptive statistics were calculated to determine the mean values and standard deviations for all shade and microhardness measurements. Intragroup comparisons of shade and microhardness across different time points were analyzed using repeated measures ANOVA with Bonferroni correction and post hoc analysis for comparison between the two groups. The paired *t*-test was employed to compare microhardness values between matched samples

from the two groups at different time points. Additionally, the Mann-Whitney U test was applied to compare surface area, pore size, and nanopore volume between the two groups. The shade parameters of the examined teeth were tested for normality (Gaussian distribution) using the Shapiro-Wilk test, and Pearson correlation analysis was performed to assess the association between the baseline shade and the change in shade produced by the treatment. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Changes in tooth shade

Changes in tooth shade and hardness following the different treatments are shown in Fig. 2. HP treatment resulted in a significant increase in both lightness (ΔL) and chroma (ΔC) parameters immediately after treatment. This increase was continuous within the first post-bleaching week. However, after this time point the chroma stabilized, while the lightness actually presented a relative decreased followed by a subsequent stabilization. The group treated with DW showed no changes in tooth shade overtime. Significant differences between the HP and DW group were noted for ΔL , ΔH , and ΔC at all time points (14 h, 1 week, 2 weeks, 3 weeks, and 4 weeks) (Fig. 1). Furthermore, it was observed that the baseline shade was negatively correlated with post-bleaching ΔL ,

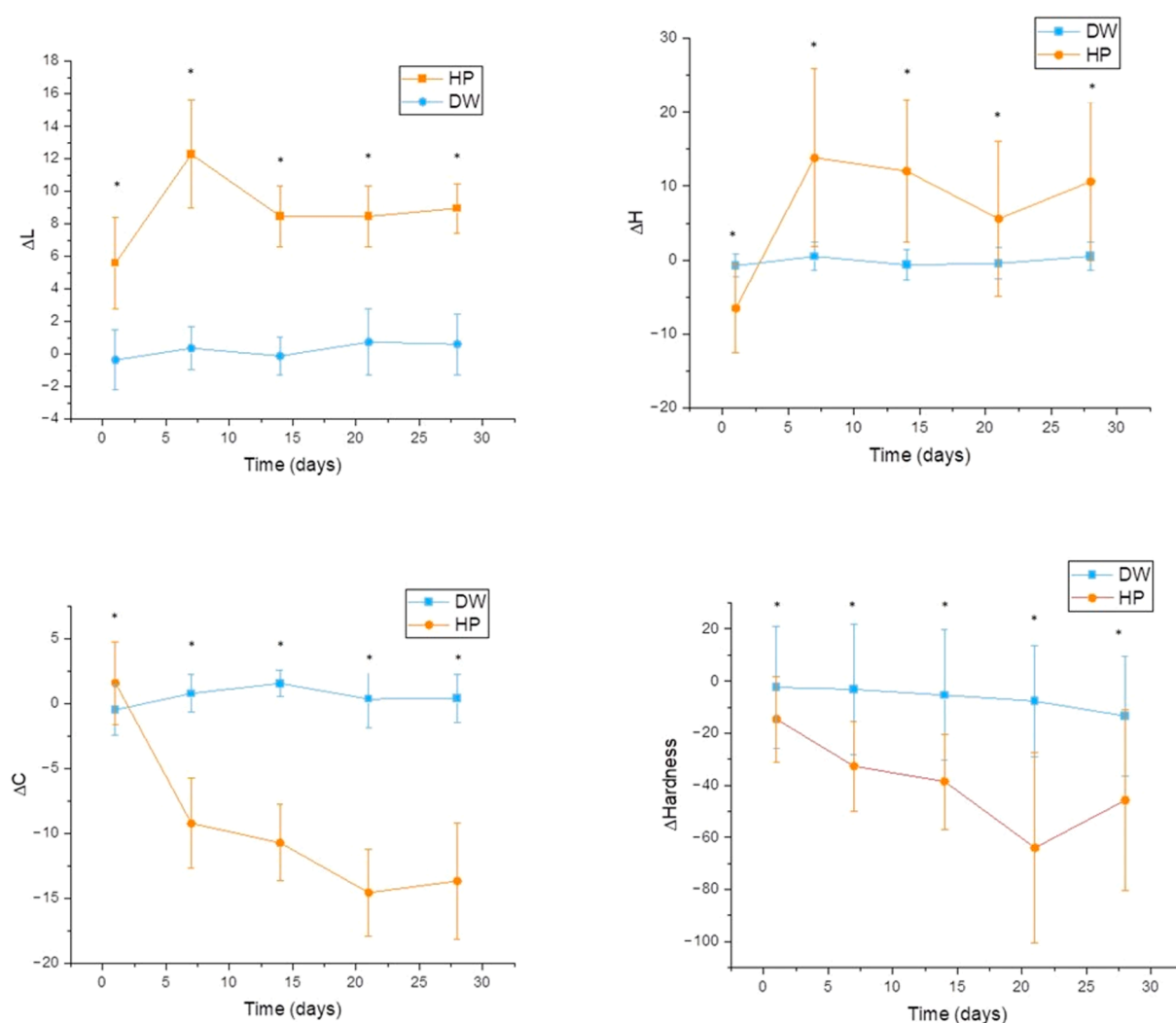


Fig. 2. Differences in ΔL , ΔH , ΔC and $\Delta \text{Hardness}$ between the bleached (HP) and control (DW) groups after 14 h, 1 week, 2 weeks, 3 weeks and 4 weeks; * indicates statistically significant differences ($p < 0.01$) in ΔL , ΔH , ΔC and $\Delta \text{Hardness}$ between the groups.

ΔH , and ΔC . In other words, lower baseline scores for shade parameters were associated with larger changes in lightness, hue, and chroma due to bleaching (Table 1). Significant negative correlations were found between baseline tooth shade and post-bleaching ΔL at the early time point, as well as between baseline tooth shade and post-bleaching ΔH at 14 h and the later time points (Fig. 3).

3.2. Changes in tooth enamel microhardness

HP treatment resulted in a continuous decrease in enamel microhardness that went on for 20 days after treatment (Fig. 2). At 14 h after treatment, the differences in microhardness between the HP and DW groups were not significant, however, at week 1 and onwards the differences were consistently significant ($p < 0.05$) (Supplemental Table 1).

3.3. Changes in protein structure

The size-distribution curve from the suspension of proteins extracted from the bleached and DW specimens is shown in Fig. 4A. The average protein size was smaller in the bleached group compared to the distilled water group.

The CD spectra of protein extracts from human teeth specimens exposed to bleaching and DW are shown in Fig. 4B. The negative ellipticity near 200 nm indicated that most protein molecules from the control group were primarily in a random coil or unstructured form. In contrast, the slight negative ellipticity observed for proteins extracted from teeth treated with HP suggested that these molecules were more likely to be folded into a structured or non-random conformation. The slightly positive band near 216 nm for the bleached group and 230 nm for the control group confirmed the presence of polyproline II (PP II) type structures in the extracts. Additionally, an analysis of the globular protein secondary structure revealed that Helices 1 and 2 were clearly observed in the control group but dissipated in the HP group. Furthermore, the frequency of turns was higher in proteins extracted from the bleached specimens compared to those from the distilled water specimens (Table 2).

3.4. Changes in surface area and pore size

According to the BET analysis, HP treatment resulted in an increase in surface area and a reduction in average pore size compared to DW treatment. Additionally, there was a slight increase in total nanopore volume after bleaching (Table 3).

Table 1
Pearson correlation analysis between the initial tooth shade and post-bleaching ΔL , ΔH and ΔC at different time points (14 h, 1 week, 2 weeks, 3 weeks and 4 weeks).

Time point	r^l	p value	r^h	p value	r^c	p value
14 h	-0.690 *	$p < 0.05$	-0.835 *	$p < 0.05$	-0.469	$p > 0.05$
1 week	-0.709 *	$p < 0.05$	-0.215	$p > 0.05$	-0.461	$p > 0.05$
2 weeks	-0.404	$p > 0.05$	-0.844 *	$p < 0.05$	-0.334	$p > 0.05$
3 weeks	-0.213	$p > 0.05$	-0.723 *	$p < 0.05$	-0.211	$p > 0.05$
4 weeks	-0.302	$p > 0.05$	-0.200	$p > 0.05$	-0.615	$p > 0.05$

r^l -Pearson correlation coefficients between baseline tooth shade and post-bleaching ΔL (lightness) at 14 h, 1 week, 2 weeks, 3 weeks and 4 weeks; r^h -Pearson correlation coefficients between baseline tooth shade and post-bleaching ΔH (hue) at 14 h, 1 week, 2 weeks, 3 weeks and 4 weeks; r^c Pearson correlation coefficients between baseline tooth shade and post-bleaching ΔC (chroma) at 14 h, 1 week, 2 weeks, 3 weeks and 4 weeks;

* $p < 0.05$ indicates that the correlation is significant.

4. Discussion

The present study confirmed the hypothesis, demonstrating that HP tooth whitening treatment denatured tooth enamel proteins and increased enamel porosity, leading to changes in enamel shade and microhardness over time.

Our study found that HP treatment led to an increase in the lightness and chroma shade parameters of teeth immediately after treatment and up to 1-week post-bleaching, after which these parameters slightly decreased. Similar findings were reported in previous studies [30,38,39]. Matis et al. [39] reported a 51 % color rebound rate one week after treatment, while de Almeida et al. [38] noted partial color rebound one week following bleaching. In contrast, Ozdemir et al. [30] observed that lightening effect continued to increase for up to two weeks after the bleaching treatment. Since the whitening of teeth is likely caused by the oxidation of enamel proteins, the shade rebound that is typically observed could be related to the loss of oxidized proteins. Indeed, previous studies have shown that enamel experiences a progressive loss of enamel protein matrix following HP treatment [13].

Furthermore, a statistically significant difference was observed between the bleached and control groups for all color parameters (ΔL , ΔH and ΔC) over time, which is closely aligned with previous studies [6,20,38]. This can be attributed to HP's ability to oxidize the enamel's protein matrix, transforming it into a more opaque and whiter material [4]. According to the literature, numerous factors can influence the effectiveness of tooth bleaching and the occurrence of color relapse, including the pH and viscosity of the bleaching agent, HP concentration, the contact time with the dental structure, light activation, number of applications and baseline tooth color [15,16,20,21,30,40,41]. In our study, we also observed that baseline tooth shade was associated with the outcome of the bleaching procedure. Teeth with darker baseline shades tended to experience more pronounced whitening than those with lighter baseline shades. Previous studies have shown that while the inherent whiteness of a tooth largely depends on the crystallographic properties of enamel [42], whitening through bleaching occurs through the oxidation of enamel proteins [4]. However, future studies will be required to explain why darker teeth show better results.

Our results revealed a continuous reduction in surface microhardness following treatment with HP. However, this reduction in microhardness became significant only 1 week after treatment, and it grew larger over time. The effect of HP on enamel microhardness is controversial in the literature, with some studies reporting findings similar to ours [17,18,30], while others have reported that HP does not significantly affect enamel microhardness [14–16]. Our findings suggest that the inconsistency observed in the literature may, in part, be related to the timing of microhardness measurements. Our results indicate that HP has a delayed effect on enamel microhardness, meaning the true magnitude of its impact on enamel can only be fully appreciated weeks after treatment.

Our results indicate that the decrease in microhardness following peroxide treatment was likely due to changes in its microstructure (i.e., porosity and surface area), which were probably caused by the degradation of the enamel organic matrix. This is supported by BET, DLS, and CD analyses, which revealed that HP treatment caused proteins to shrink and denature, as well as increased enamel surface area and nanoporosity volume fraction. The delayed effect of HP on microhardness could be related to the progressive loss of the enamel protein matrix [13]. Since the proteins plays a crucial role in binding hydroxyapatite crystals together, its disruption can increase the enamel's susceptibility to loss of hardness [5,13].

Our study observed a slight increase in surface area and pore volume after HP treatment, accompanied by a reduction in the average pore size. Similar observations were made by Orilisi et al. (2021), who used SEM to show that teeth treated with HP exhibited slight changes in surface structure, including the formation of micro porosities [43]. Other studies have shown that HP gels increased the surface roughness and

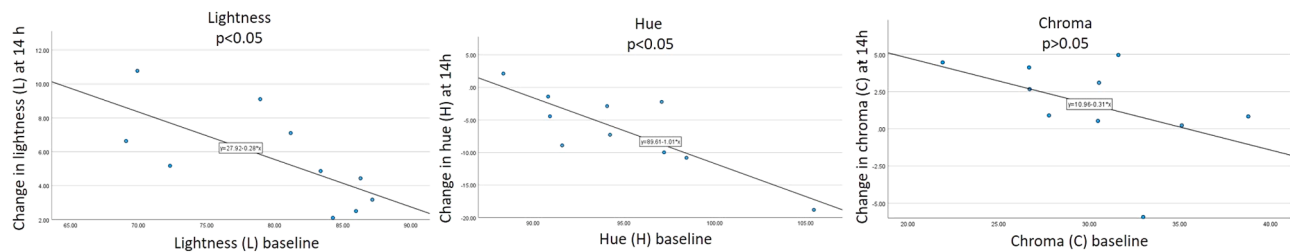


Fig. 3. Correlation analysis between the baseline tooth shade and post-bleaching Δ L, Δ H and Δ C at 14 h; $p < 0.05$ indicates statistically significant association between the baseline L, H and C and post-bleaching Δ L, Δ H and Δ C at 14 h.

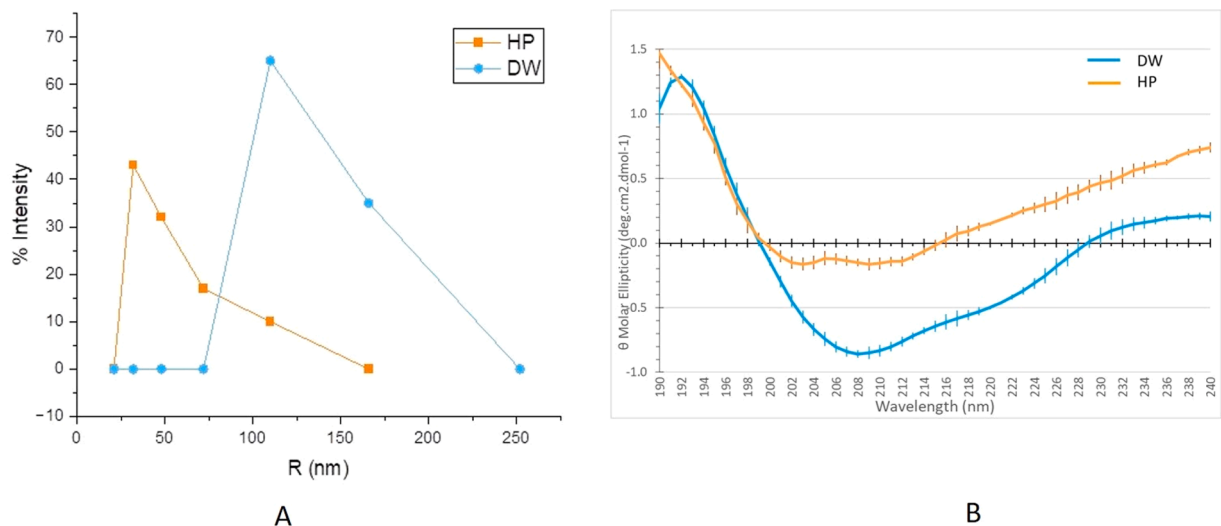


Fig. 4. (A). Dynamic light scattering protein size distribution between the bleached (HP) and control (DW) groups. (B). Circular dichroism spectra of protein extracts from the bleached (HP) and distilled water (DW) exposed human teeth.

Table 2
Estimation of the globular protein secondary structure from circular dichroism after treatment with either HP or DW.

Groups	Helix 1	Helix 2	Strand 1	Strand 2	Turns	Unordered	Total
HP	0.046*	0.001*	0.261	0.107	0.175	0.411	1.001
DW	0.149	0.010	0.177	0.057	0.167	0.440	1

* significantly different from DW group; HP-hydrogen peroxide; DW-distilled water.

Table 3
The means and standard deviations (SD) of the BET surface area, average pore size and pore volume in the bleached (HP) and control (DW) groups.

Measurements	Groups		p value ^a
	DW Mean (SD)	HP Mean (SD)	
Surface Area (m ² / g)	22.69 (1.41)	26.34 (3.46)	$p = 0.12$
Pore size (nm)	50.60 (4.84)	48.84 (0.17)	$p = 0.51$
Nanopore volume (cm ³ / g)	0.0041 (0.00055)	0.0050 (0.00052)	$p = 0.04^*$

^a p values for Mann-Whitney U test comparing the HP and DW groups;
* indicates that there is a statistically significant differences between the groups; BET-Brunauer-Emmett-Teller; DW-distilled water; HP-hydrogen peroxide.

leading to the exposure of prism rods [23,24]. In contrast, other studies have shown that gels containing 7.5 % or 35 % HP produced a surface topography similar to that of non-bleached specimens [16].
DLS and CD analysis revealed that enamel proteins in the bleached specimens were smaller compared to those in the DW samples and exhibited a loss of helical structure. These findings indicate denaturation

of the enamel proteins, which may result from a combination of random fragmentation, as shown in other studies [13], as well as protein reconfiguration, as indicated by CD in our study— a highly effective technique for analyzing protein conformations [44]. Our results demonstrated that the majority of protein molecules in the DW group were random coil/unstructured in nature, with a high helical content. In contrast, HP treatment caused the enamel protein to adopt a more structured conformation. Notably, the prominence of Helix 1 and Helix 2 was significantly reduced following HP treatment compared to the control group. This change could be attributed to the denaturation of amelogenin, a key enamel protein with a helical structure. Amelogenin molecules are organized into oligomers that form nanospheres and ribbons, creating a framework for hydroxyapatite crystal deposition [45, 46]. Denaturation of amelogenin disrupts this framework, impairing the organization of the enamel matrix. Additionally, HP may have also altered the C-terminus of amelogenin, which plays a crucial role in facilitating the binding of proteins to the mineralized matrix, leading to a loss of enamel hardness [47]. Furthermore, denatured proteins can increase enamel porosity, which can affect light scattering and absorption, potentially altering the tooth’s perceived color [30]. The changes observed in enamel structure following HP treatments could help

explain the tooth whitening effect associated with bleaching products, as well as the increase tooth sensitivity observed clinically in patients that have undergone tooth bleaching treatments [48]. These observations could also predict an increased risk of tooth erosion and wear, although this possibility would need to be investigated in future studies.

In vitro studies have inherent limitations in accurately replicating clinical conditions due to their inability to mimic the complex and dynamic environment of the human mouth. A limitation of this in vitro study was that in the aging experiments, the tooth samples were stored in distilled water rather than in natural saliva. This limitation could impact the generalizability of the results, as saliva plays a key role in the buffering and remineralization processes, and its absence may not fully replicate the reality of the environment of the oral cavity. Additionally, while the temperature in the oral cavity remains relatively constant and close to body temperature, in vitro studies may use varying temperatures, which can lead to differences between the study results and the actual performance of whitening treatments in natural conditions. In this study, we used solutions of pure HP as provided by the supplier to avoid potential sources of bias. However, commercial HP could contain additives and active ingredients that could potentially minimize the changes in the dental substrate observed in our study, although future research is needed to clarify the effects of these additives on enamel structure. In order to allow for better control over the application of tooth whitening treatments, bleaching products used both at home and in clinical practice often consist of HP gels rather than solutions. Experiments in our study were limited to HP solutions to minimize the possible confounding that could be introduced by the wide range of chemicals included in gel formulations. However, further research would be needed to clarify the role of gelling agents on the chemical effects of HP on tooth enamel.

Another limitation of our study is that we only assessed a relatively high concentration of HP. Even though the concentration assessed in our study is commonly used in clinical practice, lower concentrations are also used in clinical and homecare products (3–35 %). Other studies have investigated different concentrations of HP, but yielded conflicting results regarding the relation of HP concentration with enamel microhardness [49,50]. Thus, the effect of a wider range of HP concentrations on enamel proteins should be investigated in future studies. Furthermore, this study included various types of teeth commonly treated in bleaching procedures (upper anterior teeth and premolars). However, by including different types of teeth, this clinically relevant approach might have increased variability in our results. Future studies will also be needed to better understand the underlying causes of the unpredictability of tooth whitening treatments, and to explain why their whitening effects are often reversible and tends to be more pronounced in darker teeth.

5. Conclusion

HP tooth whitening treatment alters the tooth enamel proteins, reducing their size and leading to a shift from a non-random coil to well-ordered secondary structures. Additionally, HP increased both the enamel surface area and the volume of pores and significantly reduced enamel microhardness overtime.

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CRedit authorship contribution statement

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Formal analysis. **Ovidiu Ciobanu:** Writing – original draft, Methodology, Investigation, Formal analysis. **Mohamed-Nur Abdallah:** Writing – review & editing, Methodology, Investigation. **Valentin Dan Nelea:** Writing – review & editing, Investigation. **Nidhi Gupta:** Writing – original draft, Investigation. **Ahmed Abotaleb:** Investigation. **Faleh Tamimi:** Writing – review & editing, Resources, Methodology.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jdent.2025.105714](https://doi.org/10.1016/j.jdent.2025.105714).

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