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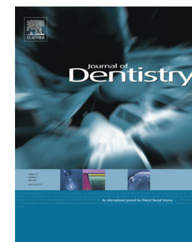

Effect of different storage conditions on the physical properties of bleached enamel: An *in vitro* vs. *in situ* study

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ABSTRACT

Objectives: Evaluate the effect of different storage conditions on bleached enamel using Knoop microhardness (KHN) and colour variation.

Methods: Forty-eight tooth blocks were divided into four groups ($n = 12$), based on storage media (SM): purified water (PW), artificial saliva (AS), natural saliva (NS), *in situ* (IS). Three whitening sessions were carried out using 35% hydrogen peroxide, with a week interval. Colour and KHN measurements were taken before the samples were placed in the SM (t_1), after 24 h in the SM (t_2), and after 24 h at the end of the bleaching treatment (t_3). Two extra samples from each group were analysed using a scanning electron microscope (SEM). KHN results were analysed by PROC-MIXED and Tukey–Kramer test ($\alpha = 0.05$), and colour changes were evaluated using ΔL, Δa, Δb, ΔE between the different times $\Delta 1(t_1 - t_2)$, $\Delta 2(t_2 - t_3)$ using the Kruskal–Wallis test and Dunn's test ($\alpha = 0.05$).

Results: Significant statistical difference was noted in KHN at t_3, with the lowest values found for PW. As for colour analysis in $\Delta E2$ and $\Delta b2$, IS showed values that were statistically lower when compared to AS. Likewise, there were differences between PW and AS in relation to IS when evaluating $\Delta L2$. In addition, NS showed similar values to IS.

Conclusions: The storage conditions had different effects on the physical properties of bleached enamel. NS was the only SM that showed similar behaviour to IS.

Clinical Significance: NS proved an effective SM in the protection and recovery of damage caused by bleaching and is a viable SM for *in vitro* studies.

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1. Introduction

Tooth bleaching is an efficient and conservative aesthetic treatment for discoloured teeth. The procedure uses hydrogen peroxide, which penetrates into the dental structure and releases free radicals. These free radicals oxidize pigment molecules, turning them into less complex molecules, leading to a whitened aspect of the teeth.[1–3]

Although there are no doubts with regards to the effectiveness of in-office bleaching, the safety of this technique on the tooth structure has been questioned.[4] Negative effects have been associated with dental bleaching and could be related to the pH value, oxidative effect or composition of the bleaching agents.[5] Some studies have evaluated the effects of tooth whitening and reported alterations in enamel morphology,[5–7] reduction in surface hardness,[8,9] enamel demineralization,[10] and changes in the chemical composition of the tooth[11]; however, other studies have not found changes to enamel structure.[12–18]

The inconsistency in the outcome of these studies might be due to differences in study design, type of storage condition, evaluation time, different concentrations of bleaching products, application time, or the pH of the bleaching agents.[18,19] Furthermore, the few studies that do show negative effects on dental properties generally have some limitations in the *in vitro* methodologies. These include the use of purified water and artificial saliva as storage conditions, which do not accurately reflect the clinical situation.[20] The clinical situation can be achieved using *in situ* studies, which are an intermediate stage between laboratory experiments and clinical trials by reproducing the clinical conditions and performing the analysis outside the oral cavity.[3]

The purpose of this study was to evaluate the effect of different storage conditions using *in vitro* (purified water, artificial saliva and natural saliva) *vs. in situ* methodology on bleached enamel structure, as evaluated using surface microhardness and colour analysis variation. The null hypotheses were: (1) the different storage conditions would have no effect on enamel microhardness and (2) the different storage conditions would have no influence on tooth colour change.

2. Materials and methods

This study was approved by the Institutional Ethics Committee (034/2014).

2.1. Sample preparation

Freshly extracted bovine incisors which were devoid of stain, enamel cracks or fractures were selected and stored in 0.1% thymol solution at 4 °C until required for use. Enamel-dentine blocks (4 mm × 4 mm) were obtained from the buccal surface, using a diamond cutting disc (4″ × 012 × 1/2, Buehler, IL, USA) coupled to a metallographic saw (Isomet 1000; Buehler, Lake Buff, IL, USA). Enamel and dentine thicknesses were standardized (1 mm enamel and 1.75 mm dentine). The dentine surface was flattened and the enamel surface was ground flat with sequential water-cooled silicon carbide paper discs (500-, 1000-, and 2000-SiC – Buehler, Lake Buff, IL, USA). Next, the blocks were polished using diamond paste (1 μm, 1/4 μm) and polishing cloths, and rinsed with running distilled water to remove debris between each stage and at the end of the whole process. All specimens were immersed in distilled water and ultrasonicated for 15 min to remove residual particles and the smear layer. Each specimen was marked with a diamond bur #1012 (KG Sorensen) on one side to standardize the sample position in the spectrophotometer. The dental blocks were sterilized with ethylene oxide and stored in sterilized distilled water at 4 °C until required for use. Forty-eight samples were used for the microhardness and colour analyses, and eight samples were used for the SEM analysis.

2.2. *In situ* aspects

Six volunteers (three male and three female), between 23 and 27 years-old, participated in the study after signing an informed consent form. These volunteers all fulfilled the inclusion criteria (absence of dental caries and/or periodontal disease, normal saliva flow) without violating the exclusion criteria (unsatisfactory restorations and prostheses in mouth, use of orthodontic appliances, use of drugs that affect salivary flow and smokers). A full-arch maxillary impression was obtained for each volunteer and a stone cast mould was fabricated. Palatal devices were made of acrylic resin containing 4 × 4 × 2.75 mm reservoirs. The palatal devices contained two reservoirs for four volunteers, and three reservoirs for two volunteers, with an added reservoir for the SEM sample. All specimens were fixed to the palatal device using sticky wax.

2.3. Saliva collection

The natural saliva used in this study was provided by the volunteers and was collected from the same individuals, at the same time of day (8:00 am), before breakfast and any oral hygiene maintenance. Salivary flow was stimulated by chewing paraffin wax (Parafilm M, American National Can, Chicago, IL) and the saliva was collected in falcon tubes retained inside a beaker filled with ice. The collected saliva was then clarified by centrifugation (JOUAN MR23i Benchtop High Speed Centrifuge Thermo Scientific MR23i, Waltham, MA, USA) at 3.800 g for 10 min at 4 °C. Next, the saliva supernatant was sterilized by filtration with a filter membrane, with a pore size of 0.22 μm using vacuum filtration systems (TPP Rapid Filtermax Vacuum Filtration Systems, Switzerland). The whole processed saliva was divided into aliquots for daily use and immediately frozen (−80 °C) until needed for use. For each daily exchange of saliva during the experiment, the aliquots of natural saliva ware thawed and mixed before use. The artificial saliva used in this study contained 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.1 mol/L Tris buffer, and pH 7.0.[21]

2.4. Peroxidase assay in natural saliva

To ensure that the salivary enzyme peroxidase remained active in natural saliva during storage, an enzyme activity test was carried out at different times, that is, after the salivary

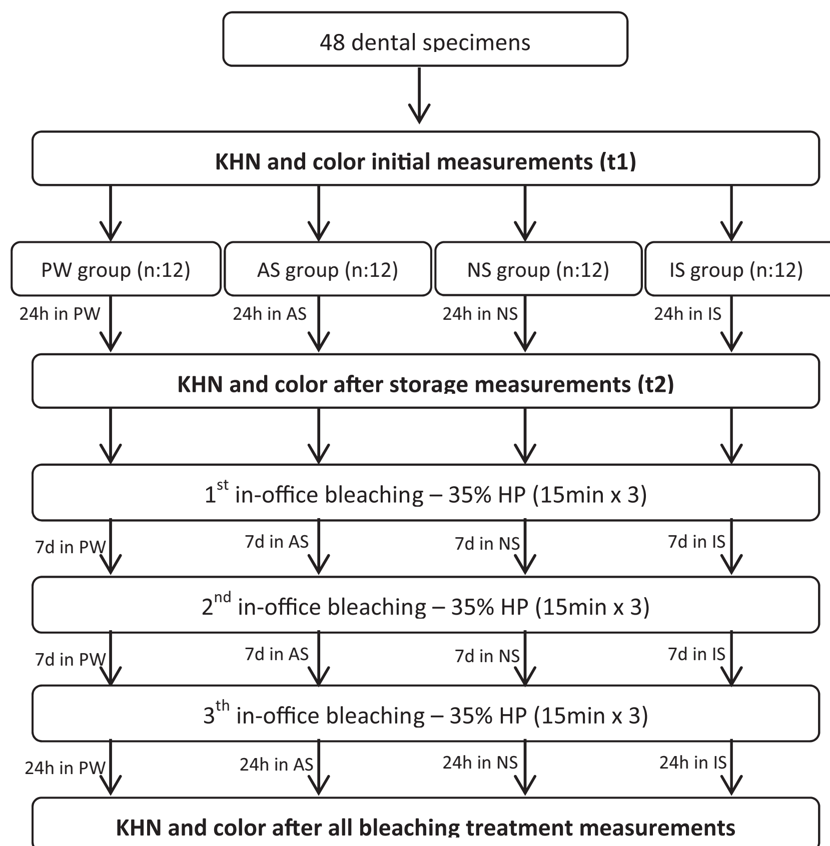

Fig. 1 – Flowchart of the experimental procedures.

processing, immediately after thawing, and after 6, 12 and 24 h storage at 37 degrees. This experiment spectrophotometrically measured (DU 800 UV/Visible spectrofotometer, Beckman Coulter, Inc., Brea, CA, USA) the oxidation of 4-amino-antipyrine, in 3 mL of 0.3 M phosphate buffer, pH 7.4, containing 10 mm hydrogen peroxide, 20 mm M 4-amino-antipyrine, 0.15 M phenol and 40 μL of stimulated natural saliva.[22] The change in absorption at 510 nm (ΔA/min) was recorded over a period of 10 min, and a unit of activity was defined by changing the absorbance of 0.001 min^{-1}. The peroxidase assay was performed in duplicate.

2.5. *Baseline measurements*

2.5.1. *Knoop microhardness*

Microhardness (KHN) was measured using a microhardness tester (Shimadzu HMV-2000 Micro Hardness Tester; Shimadzu Corporation, Kyoto, Japan) with a Knoop indenter, under 50 g load for 5 s. Five indentations, 50 μm apart, were performed on every specimen. Blocks that showed an individual average during the initial analysis that was superior or inferior than 10% of the average hardness calculated for all blocks were excluded from the experiment and substituted by another specimen fitting these parameters. After this analysis performed at the baseline when the samples were stored in purified water (initial), another two measurements were performed after 24 h storage (after storage) and 24 h after the bleaching treatments were finished (after bleaching) (Fig. 1). The average KNH at each time was calculated.

2.5.2. *Colour measurements*

A spectrophotometer (Konica Minolta CM-700d-Konica Minolta Investment Ltd. Sensing Business Division, Shanghai, China) was used to measure the colour of the tooth, based on the CIE $L^*a^*b^*$ colour space system. Colour measurement was performed three times: baseline, when the samples were stored in purified water (initial/t_1), after 24 h storage (after storage/t_2), and 24 h after the bleaching treatments were complete (after bleaching/t_3) (Fig. 1). The specimens were placed in a Teflon device (sample holder) inside a light cabin (GTI Mini Matcher MM1e, GTI Graphic Technology Inc., Newburgh, NY, USA) with a daylight lamp to standardize the ambient light during the measurement process. The spectrophotometer was previously calibrated and used in accordance with the manufacturer's instructions. The colour of the samples was measured and quantified in terms of the L^*, a^*, b^* coordinate values established by the *Commission Internationale de l'Eclariage* (CIE), in which the L^* axis represents the degree of lightness within a sample and ranges from 0 (black) to 100 (white). The a^* coordinate represents the degree of green/red colour, and the b^* coordinate represents the degree of blue/yellow colour. The measurement of colour change in the different times was made by calculating the variation of L^* ($\Delta L1 = L^*_{after\ storage} - L^*_{initial}$ and $\Delta L2 = L^*_{after\ bleaching} - L^*_{after\ storage}$), a^* ($\Delta a1 = a^*_{after\ storage} - a^*_{initial}$ and $\Delta a2 = a^*_{after\ bleaching} - a^*_{after\ storage}$), and b^* ($\Delta b1 = b^*_{after\ storage} - b^*_{initial}$ and $\Delta b2 = b^*_{after\ bleaching} - b^*_{after\ storage}$) and the total colour change (ΔE) was calculated according to the following formula[23]: $\Delta E = \sqrt{[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]}$.

2.5.3. Scanning electron microscopy (SEM)

After the bleaching treatments, two extra samples from each group were dehydrated by immersion in different alcohol concentrations (50%, 75%, 90% and 100%) fixed with double-sided carbon tape on stubs, and sputter-coated with gold for SEM analysis. The analysis was performed using a scanning electron microscope (JEOL JSM-5600 LV, Tokyo, Japan) with 1000× of magnification. The representative areas of each group were photographed for comparison.

2.5.4. Pre-bleaching preparation

After baseline measurements (initial), the dental blocks were randomly divided into four storage condition groups (*n*: 12): purified water (PW), artificial saliva (AS), natural saliva (NS) and *in situ* (IS). Once the *in situ* specimens were fixed in the palatal devices, they were kept in the oral environment of the volunteers for one day to form salivary pellicle. During the intraoral exposure period, the samples were cleaned with water and a soft toothbrush, which was provided to the volunteers in advance, without any dentifrice. The palatal devices were removed only for meals in order to exclude the effects of food components on pellicle development, and were stored in a humid environment during this time.

Twenty-four hours after all of the study specimens were in their storage condition groups, the second analyses of colour and microhardness were carried out (after storage), preceding the bleaching treatment.

2.5.5. Bleaching procedure

The in-office bleaching procedure initiated after one day of storage. The specimens were taken from the storage environment and wiped with absorbent paper. Next, 35% hydrogen peroxide (Whiteness HP MAXX; FGM Odontology Products, Brazil) was applied three times for 15 min, totalling 45 min of gel application. The bleaching gel was removed using flexible plastic cotton-tipped rods, washed thoroughly with distillated water and stored in each corresponding storage condition group. The pH of the bleaching gel was assessed using a calibrated pHmeter (Procyon, digital pH metre model AS 720, Procy Instrumental Científica, São Paulo, SP, Brazil) and was found to be 5.45. The *in vitro* groups were stored in 2 mL of storage solution (PW, AS, NS), at 37 degrees, which was changed daily. For the *in situ* group, the palatal devices were returned to the volunteers' oral cavities after bleaching, and remained there during the whole experiment. Three bleaching sessions were held with a seven-day interval between the sessions. Twenty-four hours after the third bleaching session, the final measurements of colour and microhardness were performed (after bleaching). The three time analyses of the experiment are shown in Fig. 1.

2.6. Statistical analysis

The microhardness measurements were analysed using the methodology of mixed models for repeated-measures using the PROC MIXED procedure of SAS statistical software, and the multiple comparisons were performed using the Tukey–Kramer test considering a level of significance of 5%. The colour data, ΔL, Δa, Δb, ΔE between the different times $\Delta 1$ ($t_1 - t_2$) and $\Delta 2$ ($t_2 - t_3$) were analysed using Kruskal–Wallis tests, followed by the Dunn test ($\alpha = 0.05$).

3. Results

3.1. Peroxidase assay in natural saliva

Fig. 2 shows peroxidase enzyme activity in the following: natural saliva, after processing (t_0), after being thawed (t_1), and after storage at 37 °C for 6 h (t_2), 12 h (t_3) and 24 h (t_4). Peroxidase activity was proven at all five time analyses.

3.2. Knoop microhardness

Mean microhardness values are shown in Table 1. The repeated-measures by PROC MIXED followed by Tukey–Kramer test showed statistically significant results for time factor ($p = 0.0002$), for storage condition factor ($p = 0.0025$) and for the interaction of storage condition and time ($p < 0.0001$).

3.3. Colour measurements

Tables 2–5, respectively, show colour variation between initial and after storage ($\Delta 1$), and after storage and after bleaching ($\Delta 2$), of ΔE, Δa, Δb, and ΔL.

In relation to the overall variation of colour, $\Delta E1$ showed no significant statistical difference between groups. However, at $\Delta E2$, IS showed significant differences from AS. The Δa analysis demonstrated that the only difference found was when comparing NS to the other groups for $\Delta a1$. When analysing the yellowness (Δb), a significant statistical difference occurred for IS at $\Delta b1$, with higher values in relation to the other groups. At $\Delta b2$, differences were noticed only between IS and AS. The measurement of brightness variation at $\Delta L1$ indicated no difference between groups. Nevertheless, there were differences between IS when compared to PW and AS at $\Delta L2$. In addition, IS showed similar values to NS.

3.4. Scanning electronic microscopy

Demineralization of periphery enamel prisms was observed in the photomicrographs of PW. No alterations to the enamel surface were observed in the photomicrographs for the AS, NS and IS groups (Fig. 3).

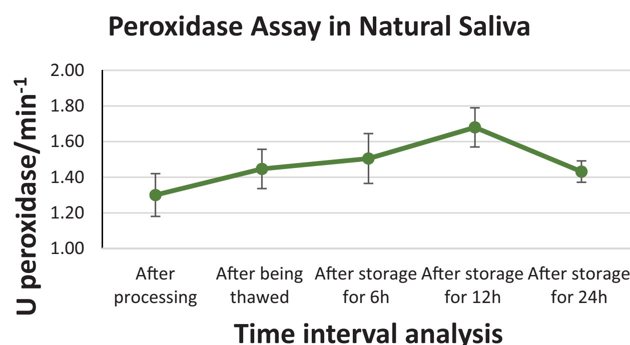

Fig. 2 – Analysis graph of peroxidase activity.

Table 1 – Mean and standard deviation (SD) of Knoop microhardness.

Storage condition	KHN (initial)	KHN (after storage)	KHN (after bleaching)
Purified water	336.9 (18.96) Aa	332.7 (18.24) Aa	279.9 (17.28) Bb
Artificial saliva	336.8 (21.38) Aa	331.1 (17.02) Aa	332.0 (16.79) Aa
Natural saliva	333.4 (14.69) Aa	338.9 (13.27) Aa	337.8 (13.89) Aa
In situ	327.5 (22.54) Aa	325.1 (12.17) Aa	338.3 (19.72) Aa

Means followed by different letters (uppercase horizontally and lowercase vertically) show statistical differences ($p \leq 0.05$).

4. Discussion

Based on the current analyses, the null hypotheses tested in this investigation were rejected. The KHN results showed a reduction in surface microhardness in the samples stored in purified water, after bleaching. However, the samples maintained in the other storage media showed no decrease in hardness at the same time analyses.

Table 2 – Median (minimum/maximum) values of ΔE.

Storage condition	ΔE1	ΔE2
Purified water	0.64 (0.10/2.75) a	6.62 (4.78/10.47) ab
Artificial saliva	0.66 (0.17/1.80) a	7.72 (4.89/8.95) a
Natural saliva	0.74 (0.30/3.27) a	5.43 (4.01/8.79) ab
In situ	0.61 (0.25/1.14) a	4.86 (2.90/8.70) b

Medians followed by different letters in the columns indicate statistical differences ($p \leq 0.05$).
$\Delta L1 = L^*_{\text{after storage}} - L^*_{\text{initial}}/\Delta L2 = L^*_{\text{after bleaching}} - L^*_{\text{after storage}}$.

Table 3 – Median (minimum/maximum) values of Δa.

Storage condition	Δa1	Δa2
Purified water	0.17 (−0.05/0.36) b	−0.71 (−1.21/−0.40) a
Artificial saliva	0.10 (0.05/0.37) b	−0.44 (−0.88/−0.15) a
Natural saliva	0.33 (0.18/0.69) a	−0.68 (1.55/−0.19) a
In situ	0.07 (−0.11/0.56) b	−0.55 (−1.30/0.26) a

Medians followed by different letters in the columns indicate statistical differences ($p \leq 0.05$).
$\Delta a1 = a^*_{\text{after storage}} - a^*_{\text{initial}}/\Delta a2 = a^*_{\text{after bleaching}} - a^*_{\text{after storage}}$.

Table 4 – Median (minimum/maximum) values of Δb.

Storage condition	Δb1	Δb2
Purified water	−0.11 (−1.40/0.55) b	−5.61 (−8.94/−3.41) ab
Artificial saliva	−0.06 (−0.93/0.79) b	−6.80 (−7.92/−3.91) b
Natural saliva	−0.25 (−0.72/0.30) b	−4.20 (−7.65/−3.41) ab
In situ	0.39 (−0.16/1.14) a	−4.31 (−8.06/−2.20) a

Medians followed by different letters in the columns indicate statistical differences ($p \leq 0.05$).
$\Delta b1 = b^*_{\text{after storage}} - b^*_{\text{initial}}/\Delta a2 = b^*_{\text{after bleaching}} - b^*_{\text{after storage}}$.

Table 5 – Median (minimum/maximum) values of ΔL.

Storage condition	ΔL1	ΔL2
Purified water	0.05 (−1.82/2.70) a	3.65 (1.88/5.32) a
Artificial saliva	−0.33 (−1.16/1.62) a	3.11 (1.10/4.42) a
Natural saliva	−0.25 (−3.20/1.48) a	2.52 (1.84/5.35) ab
In situ	0.20 (−0.29/0.79) a	1.77 (−0.17/3.14) b

Medians followed by different letters in the columns indicate statistical differences ($p \leq 0.05$).
$\Delta b1 = b^*_{\text{after storage}} - b^*_{\text{initial}}/\Delta a2 = b^*_{\text{after bleaching}} - b^*_{\text{after storage}}$.

A reduction in hardness after bleaching has been reported in other studies[5,8,9,13,24] and has often been associated with demineralization of the surface by the action of bleaching agents, probably due to its low pH and its oxidative power.[4] In this present study, a change in hardness was observed only in PW. Purified water provides no remineralization, it only keeps the samples hydrated, which does not represent the clinical condition. In the oral cavity, saliva is supersaturated with minerals, such as calcium and phosphate, which both act in the remineralization process.[25]

Furthermore, the saliva in the oral cavity acts in the formation of the acquired pellicle. The pellicle prevents the reduction in hardness by eliminating direct contact of teeth with acidic substances; acting as a diffusion barrier for calcium and phosphate; and by protein regulation of calcium and phosphate concentrations by sthaterin, mucin, histatin and proline-rich protein.[26] The *in vitro* and *in situ* use of natural saliva in the current study may have acted to eliminate the reduction of hardness after the third session of whitening, through the previously mentioned protective properties. However, the samples maintained in artificial saliva may have kept their hardness due to a super-saturation of mineral components during storage.

Both natural saliva and artificial saliva were able to maintain the hardness after bleaching treatment when used *in vitro* and *in situ*. However, other studies that used artificial saliva as the storage medium were unable to reverse the demineralization effect.[3,9,10,24,27,28] This might have been caused by a lack of standardization in artificial saliva formulations, since different types of saliva may result in higher or lower remineralization. This lack of standardization impairs the comparison and application of various study results.[29] Another fact that may compromised the remineralization after bleaching by the artificial saliva is the presence of carboxymethylcellulose (CMC). CMC is added to some artificial saliva formulations to increase its viscosity. However, this component may reduce remineralization by forming complexes between calcium and/or phosphate ions, resulting in the unavailability of these ions for mineral replacement. In addition, the increase of artificial saliva viscosity promoted by CMC can decrease the rate of diffusion of minerals into the tooth structure.[29,30]

The demineralization that occurred in purified water is observed in Fig. 3A, which shows regions of porosity, especially on the periphery of the enamel prisms. These regions of porosity characterize the beginning of an erosive process,[31] which is in agreement with the hardness loss found in this group.

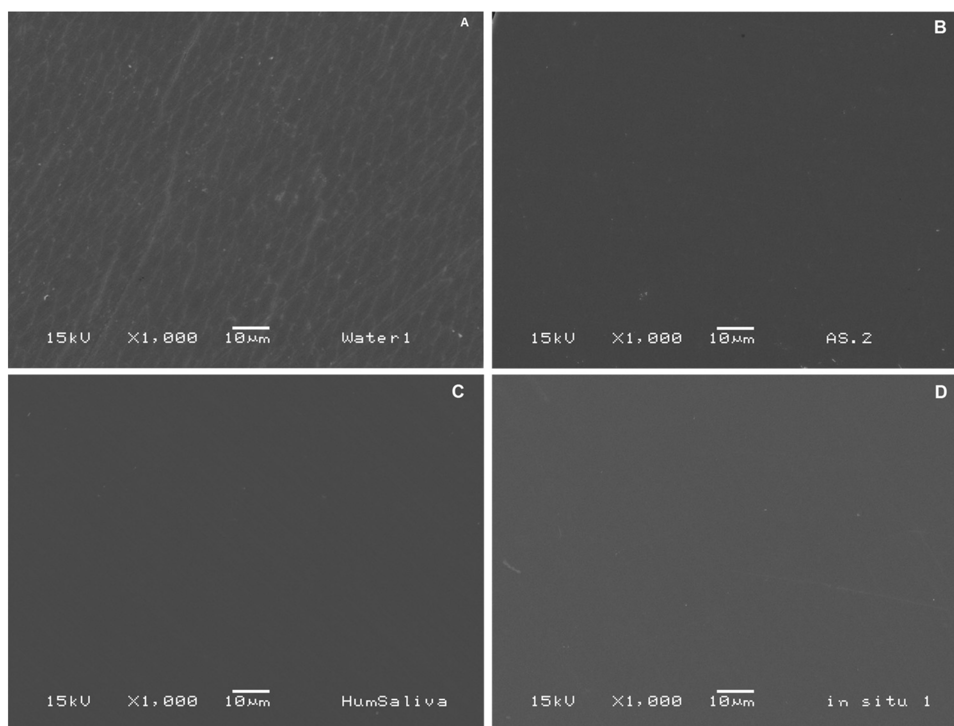

Fig. 3 – Photomicrographs of all groups 24 h after the three bleaching sessions: purified water (A), artificial saliva (B), natural saliva (C), *in situ* (D); 1000× magnification.

With regards to the colour analysis, bleaching with 35% hydrogen peroxide was effective in all storage conditions. The analysis of Δb and ΔE showed similar variations at $\Delta 2$. The AS group showed a higher colour variation in relation to IS, while the other groups did not differ from each other. When comparing the values, IS showed a reduced level of bleaching and AS presented a greater colour change.

This difference between IS and AS was not be observed in PW storage, since the reflection of light is affected by various aspects, such as: specular reflection on the surface; diffuse light reflection on the surface; absorption and scattering of light within dental tissues; and the enamel mineral content. Therefore, the PW specimens, which showed a reduction in hardness and alterations to the surface, probably reflected light differently when compared to AS.[32]

In addition, IS showed a higher variation in $\Delta b1$ as a result of an increase in the b^* value between water storage and after 24 h in the volunteer's oral cavity. This variation indicates an increase in yellowness, probably driven by a greater hydration caused by natural saliva organic components, such as statherin and mucin, which offer a greater potential for hydration and lubrication.[33–35] However, the same was not observed for $\Delta E1$, which can be justified by the fact that ΔE is an analysis that takes into account all colour coordinates, and L^* and a^* changes were not observed in this study.

In relation to Δa, a difference was observed only at $\Delta a1$, where NS showed higher values when compared to the other groups, representing a reduction green colour. This fact, in addition to the alteration in $\Delta b1$, reveals the importance of performing colour analysis after placing the samples in the storage medium and before the bleaching treatment, revealing a dependence on the colour alteration pattern based on storage medium.

The analysis of brightness provided by ΔL showed that NS was the only group that was the same for both *in vitro* groups and the *in situ* group for $\Delta L2$. On the other hand, IS differed from PW and AS and had the lowest values of change in brightness. These differences in the values of colour variation between times, mainly involving IS, might have occurred because the specimens remained within the oral cavity throughout the study time, allowing for constant contact with saliva, which has an intermittent production in the oral cavity.

The volunteers who participated in this research used an intraoral device and donated saliva. Thus, the natural saliva used was provided from the same volunteers who participated in the IS group. The saliva is very important for the protection and maintenance of enamel properties. In addition to presenting mineral components, such as calcium and phosphate that act in remineralizing teeth, saliva has many organic compounds that act for the protection and maintenance of tooth structure.[25,30] This protective function of saliva occurs through acquired pellicle formation, which contains several proteins, such as proline-rich proteins, histatins, statherin; which participate in the mineral homeostasis of calcium and phosphate by controlling the deposition of these minerals on teeth.[33,34] Moreover, statherin acts in lubricating the oral cavity,[25,35] while mucin, which is highly glycosylated, provides viscoelastic properties to the saliva.[32–34] Therefore, these organic components in the acquired pellicle might have acted like a barrier[26] against the penetration of hydrogen peroxide or provided better

maintenance of hydration of the specimens stored *in situ*, which may have contributed to the lesser whitening effect observed for *in situ* storage. However, the same may not have been observed in the NS group. This may be due to the fact that natural saliva was changed only every 24 h and was probably subjected to decomposition[29] and possible consumption of salivary components by the samples.

The presence of salivary peroxidase constitutes another factor that may have contributed to reduced whitening of the IS group, especially in the evaluation of ΔL, which measures brightness of the tooth and has great importance for dental bleaching. And this may justify the result that NS was the only group whose results were similar to those of IS.

The role of peroxidase in natural saliva is to catalyse the decomposition reaction of hydrogen peroxide.[22,36,37] Hence, it is very important that its activity was maintained in the natural saliva used *in vitro* and stored at 37 degrees. The maintenance of this activity was confirmed using a colorimetric test after processing, freezing, thawing and storage of saliva at 37 degrees for 24 h.

Hydrogen peroxide is a highly unstable molecule that can dissociate into water and free radicals. The free radicals break down the organic pigment molecules inside the tooth structure.[2] However, when this reaction is catalysed by peroxidase, a known antioxidant, there is a rapid consumption of the hydrogen peroxide molecules and the sub products of this reaction are water and oxygen, with a reduced production of free radicals.[22,38,39]

Therefore, the action of peroxidase on the bleaching gel leads to fewer free radicals breaking down the pigments inside the tooth. This causes a reduced bleaching effect, especially when increasing brightness.

5. Conclusion

The storage conditions demonstrated different results regarding the effects on the physical properties of the enamel. Thus, when *in vitro* researches search for results that approximate the clinical conditions regarding colour variation analysis, the most appropriate storage medium is natural saliva, since it was the only storage medium that showed the closest results to *in situ* results. However, *in vitro* studies to assess hardness after bleaching, both in artificial and natural saliva, were efficient in enamel remineralization.

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