Sara Tiemi Felipe Akabane

AVALIAÇÃO DE AGENTE CLAREADOR PARA USO PROFISSIONAL CONTENDO TRIMETAFOSFATO E FLUORETO SOBRE A ALTERAÇÃO DE COR, PENETRAÇÃO TRANS-AMELODENTINÁRIA, DUREZA E CITOTOXICIDADE: ESTUDO IN VITRO

Araçatuba – SP
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Orientador: Profº. Titular. Alberto Carlos Botazzo Delbem
Coorientadores: Profª. Drª. Marcelle Danelon
Profº. André Luiz Fraga Briso

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Dedico este trabalho,

A minha mãe, Cilene Felipe, em memória.

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"Somos assim. Sonhamos o voo, mas tememos as alturas. Para voar é preciso amar o vazio. Porque o voo só acontece se houver o vazio. O vazio é o espaço da liberdade, a ausência de certezas. Os homens querem voar, mas temem o vazio. Não podem viver sem certezas. Por isso trocam o voo por gaiolas. As gaiolas são o lugar onde as certezas moram."

Rubem Alves

Resumo

Akabane, STF. **Avaliação de agente clareador para uso profissional contendo trimetafosfato e fluoreto sobre a alteração de cor, penetração trans-amelodentinária, dureza e citotoxicidade: estudo *in vitro*.** 2020 56f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2020.

O objetivo do presente estudo foi avaliar a adição de trimetafosfato de sódio (TMP) e / ou fluoreto de sódio (NaF) ou gluconato de cálcio (CaGlu) a um gel clareador de peróxido de hidrogênio 35% (H_2O_2) sobre a alteração de cor, microdureza do esmalte, penetração e citotoxicidade transamelodentinária. Discos de esmalte / dentina bovinos (n = 288) foram divididos de acordo com o gel clareador: 35% H_2O_2; 35% H_2O_2 + 0,05% NaF; 35% H_2O_2 + 0,25% TMP; 35% H_2O_2 + 0,05% NaF + 0,25% TMP; 35% H_2O_2 + 0,1% NaF + 1% TMP e 35% H_2O_2 + 2% CaGlu. Os géis clareadores foram aplicados três vezes (40 min / sessão) com intervalo de 7 dias entre cada aplicação. Em seguida, a alteração de cor, porcentagem de perda de dureza de superfície (% SH), dureza em secção transversal (ΔKHN), penetração trans-amelodentinária de H_2O_2, viabilidade e morfologia celular (células odontoblastoides MDPC-23), atividade de fosfatase alcalina (ALP) e deposição de nódulos de mineralização foram determinados. Os dados foram submetidos à ANOVA seguido do teste de Student-Newman-Keuls ($p < 0,05$). Todos os géis clareadores apresentaram alterações de cor significativas após o tratamento ($p < 0,001$). A perda mineral (% SH e ΔKHN) e a penetração de H_2O_2 foram menores para 35% H_2O_2 / 0,1% NaF / 1% TMP, e 35% H2O2 / 2% CaGlu apresentaram maiores valores, em comparação com os outros grupos ($p < 0,001$). A viabilidade celular foi em torno de 9%, exceto para o gel clareador contendo 35% H_2O_2 / 0,1% NaF / 1% TMP com 12,8% ($p < 0,05$). ALP foi maior para os grupos contendo TMP em comparação com outros géis clareadores ($p < 0,05$). A formação de nódulos de mineralização foi maior nos géis contendo NaF / TMP ou CaGlu ($p < 0,05$). As alterações da morfologia celular foram intensas para todos os géis clareadores. Concluiu-se que a adição de NaF / TMP em consultório não interferiu na eficácia do clareamento e reduziu a desmineralização do esmalte, a penetração do H_2O_2 e a citotoxicidade.

Palavras-chave: Desmineralização; Citotoxicidade; Remineralização; Esmalte dentário; Fluoreto; Fosfato; Clareamento dentário.

Abstract

Akabane, STF. **Evaluation of bleaching agent for professional use containing trimetaphosphate and fluoride on color alteration, trans-amelodentinal penetration, hardness and cytotoxicity: in vitro study.** 2020 56f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2020.

Abstract

This study evaluated the addition of sodium trimetaphosphate (TMP) and/or sodium fluoride (NaF) or calcium gluconate (CaGlu) to a 35% hydrogen peroxide (H_2O_2) bleaching gel on the color change, enamel microhardness, penetration and cytotoxicity trans-amelodentinal. Bovine enamel/dentin disks (n=288) were divided according to the bleaching gel: 35% H_2O_2; 35% H_2O_2 + 0.05% NaF; 35% H_2O_2 + 0.25% TMP; 35% H_2O_2 + 0.05% NaF + 0.25% TMP; 35% H_2O_2 + 0.1% NaF + 1% TMP and 35% H_2O_2 + 2% CaGlu. The bleaching gels were applied thrice (40 min/session) at the intervals of 7 days between each application. Then, the color change, percentage of surface hardness loss (%SH), cross-sectional hardness (ΔKHN), trans-amelodentinal penetration of H_2O_2, cell viability and morphology (MDPC-23 odontoblast-like cells), alkaline phosphatase activity (ALP) and deposition of mineralization nodules were determined. The data were submitted to ANOVA followed by the Student-Newman-Keuls test ($p<0.05$). All bleaching gels showed significant color changes after treatment ($p<0.001$). Mineral loss (%SH and ΔKHN) and H_2O_2 penetration were lower for 35% H_2O_2/0.1% NaF/1% TMP, and 35% H_2O_2/2% CaGlu showed higher values, compared to the other groups ($p <0.001$). The cell viability was around 9%, except for bleaching gel containing 35% H_2O_2/0.1% NaF/1% TMP with 12.8% ($p<0.05$). ALP was higher for groups containing TMP compared to others whitening gels ($p<0.05$). The formation of mineralization nodules was greater for gels containing NaF/TMP or CaGlu ($p<0.05$). The alterations of cell morphology were intense for all bleaching gels. It was concluded that the addition of NaF/TMP in-office bleaching did not interfere in the bleaching efficacy, and reduced enamel demineralization, H_2O_2 penetration and cytotoxicity.

Keywords: Demineralization; Cytotoxicity; Dental enamel; Fluoride; Phosphate; Dental bleaching.

Lista de Abreviaturas

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ACP Amorphous calcium phosphate

ALP Alkaline phosphatase activity

APC Artificial pulp chamber

ANOVA Analysis of Variance

ºC Degrees Celsius

Ca Calcium

$CaCl_2$ Calcium Chloride

CaGlu Calcium gluconate

Ca^{+2} Calcium ion

CaF^{+} Calcium fluoride ion

$CaHPO_4^0$ Phosphate hydrogenated calcium neutral

$Ca\ (NO_3)_2.H_2O$ Calcium nitrate tetrahydrate

CM Centimeters

CIE Commissione international de I´Eclairage

F Fluoride

g Gram

h Hour

$HF^º$ Neutral hydrogen fluoride

KCl potassium chloride

KHN Knoop hardness unit

H_2O Water

H_2O_2 Hydrogen peroxide

MDPC-23 Immortalized odontobast-like cell line

MEV Scanning electron microscopy

Min Minutes

mg Milligram

ml Milliliter

mm Millimeter

mmol/l Milimol per liter

Mol/l Mol per liter

NaF Sodium Fluoride

NaOH Sodium hydroxide

$NaH_2PO_4.H_2O$ Sodium phosphate monobasic monohydrate

nm Nanometers

pH Hydrogen potential

ROSs Reative oxygen species

s Seconds

SEM Scanning electron microscopy

SHi Initial surface hardness

SH_f Final surface hardness

% SH Percentage of loss surface hardness

TMP Sodium trimetaphosphate

Lista de Figuras

Figure 1 – Means values (SD) of the alteration of: (A) lightness degree (ΔL*), (B) green/red degree (Δa*), (C) blue/yellow degree (Δb*) and total color (ΔE) by Comission Internacionale de I'Eclairage (CIE) accordint to bleaching gels and moment of analysis (n=12). Equal lowercase letters indicate that there is no difference among the bleaching gels at each moment of analysis and among the moments of analysis for each bleaching gel (Student-Newman-Keuls; $p < 0.05$).

Figure 2 – Cross-sectional hardness profiles at different depths in the enamel (n=12) according to the bleaching gels.

Figure 3 – Graphics representatives of percentage of the Cell viability (A), alkaline phosphatase activity (B) and formation of mineralization nodules (C). Different lowercase letters indicate statistical difference among groups (Student-Newman-Keuls; $p < 0.05$).

Figure 4 – Photomicrographs from scanning electron microscope (×500) representative of the negative control and bleached groups.

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Table 1 – Means values (SD) of final surface hardness (SHf), percentage of loss surface hardness (%SH), integrated area (KHN × µm) and trans-amelodentinal penetration of H2O2, according experimental gels (n=12)

Sumário

SUMÁRIO

Artigo

Evaluation of the aesthetic effect, enamel microhardness and trans-amelodentinal cytotoxicity of a new bleaching agent for professional use containing trimetaphosphate and fluoride

Short title: Bleaching gel with TMP reduces mineral loss

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ABSTRACT

This study evaluated the addition of sodium trimetaphosphate (TMP) and/or sodium fluoride (NaF) or calcium gluconate (CaGlu) to a 35% hydrogen peroxide (H_2O_2) bleaching gel on the color change, enamel microhardness, penetration and cytotoxicity trans-amelodentinal. Bovine enamel/dentin disks (n=288) were divided according to the bleaching gel: 35% H_2O_2; 35% H_2O_2 + 0.05% NaF; 35% H_2O_2 + 0.25% TMP; 35% H_2O_2 + 0.05% NaF + 0.25% TMP; 35% H_2O_2 + 0.1% NaF + 1% TMP and 35% H_2O_2 + 2% CaGlu. The bleaching gels were applied thrice (40 min/session) at the intervals of 7 days between each application. Then, the color change, percentage of surface hardness loss (%SH), cross-sectional hardness (ΔKHN), trans-amelodentinal penetration of H_2O_2, cell viability and morphology (MDPC-23 odontoblast-like cells), alkaline phosphatase activity (ALP) and deposition of mineralization nodules were determined. The data were submitted to ANOVA followed by the Student-Newman-Keuls test ($p<0.05$). All bleaching gels showed significant color changes after treatment ($p<0.001$). Mineral loss (%SH and ΔKHN) and H_2O_2 penetration were lower for 35% H_2O_2/0.1% NaF/1% TMP, and 35% H_2O_2/2% CaGlu showed higher values, compared to the other groups ($p <0.001$). The cell viability was around 9%, except for bleaching gel containing 35% H_2O_2/0.1% NaF/1% TMP with 12.8% ($p<0.05$). ALP was higher for groups containing TMP compared to others whitening gels ($p<0.05$). The formation of mineralization nodules was greater for gels containing NaF/TMP or CaGlu ($p<0.05$). The alterations of cell morphology were intense for all bleaching gels. It was concluded that the addition of NaF/TMP in-office bleaching did not interfere in the bleaching efficacy, and reduced enamel demineralization, H_2O_2 penetration and cytotoxicity.

Keywords: Demineralization; Cytotoxicity; Dental enamel; Fluoride; Phosphate; Dental bleaching.

1. Introduction

The current dentistry has been strongly affected by great aesthetic demand, with teeth whitening being one of the most required clinical procedures by patients. This treatment can be carried out using the at-home technique, which is characterized by the daily exposure to low concentration peroxides or by the in-office technique, which uses peroxides highly concentrated in the elements to be cleared [1]. Both techniques are based on the release of reactive oxygen species (ROSs), which, due to their extreme instability, end up reacting and cleaving the chromophoric agents present in the dental structure, transforming them into smaller molecules and making the teeth lighter. However, the great challenge of whitening treatment is to offer the patient a safe, fast, comfortable and satisfactory technique [2]. Some studies report that whitening agents can cause changes in enamel morphology [3,4] and decrease in enamel hardness [5,6]. Furthermore, an enamel demineralized would facilitate the ROSs penetration leading to dental sensitivity, since it can also reach the dentin-pulp complex [5,7], resulting in inflammatory responses, cellular changes and possible pulp damage [2,8]. Therefore, it is essential to seek strategies using remineralizing agents, which added to the bleaching gels, can somehow reduce or minimize mineral loss and dental sensitivity.

New agents with remineralizing potential phosphate-based associated or not to sodium fluoride (NaF) have been studied. They can be a calcium or phosphate source [5] as calcium chloride (CaCl2), calcium gluconate (CaGlu) or amorphous calcium phosphate (ACP), however without clear evidence to minimize the side effects of the bleaching agents. The other phosphate-based agents act as a barrier against acid diffusion and works as nucleators of calcium phosphate apatite-like [9-12], as the cyclophosphates. Sodium trimetaphosphate (TMP) is a cyclotriphosphate that have been demonstrated a great effect against enamel demineralization [10,13] when associated to low-fluoride toothpaste [9,10]. According to studies on enamel demineralization, TMP can assist in the mineral retention of hydroxyapatite, forming a more stable crystal [11]. Therefore, it would be valid to evaluate whether the addition of TMP to bleaching agents with a high concentration of hydrogen peroxide (H_2O_2) can inhibit mineral loss, trans-amelodentinal diffusion and pulp damage.

Based on previous studies [9], TMP would have the ability to reduce the H+ ions diffusion into the enamel, reducing demineralization, as well as it would bind to anions from H_2O_2 minimizing the dentin-pulp complex damages. However, it is necessary to verify whether TMP would not reduce the whitening ability when added to H_2O_2. Given the above, knowing the good properties

of TMP and NaF, it would be interesting to evaluate the formulation of a new bleaching gel for professional use with these agents that aims to potentiate the inhibiting effect on the enamel demineralization, maintaining the bleaching activity of the H_2O_2.

Therefore, the present study evaluated the ability of experimental gels containing 35% H_2O_2 and NaF/TMP or CaGlu to minimize the adverse effects produced by 35% H_2O_2 such as mineral loss and trans-amelodentinal cytotoxicity, without interfering with the clinical effect of tooth whitening. The null hypothesis of the study was that bleaching gels containing 35% H_2O_2 and NaF/TMP or CaGlu would promote a bleaching effect, mineral loss and trans-amelodentinal cytotoxicity similar to the bleaching gel containing only 35% H_2O_2.

2. Materials and Methods

2.1. Experimental Design

Enamel/dentin disks (5.7 mm in diameter × 3.5 mm in thickness) were obtained from bovine incisor teeth. The disk were cleaned in deionized water and stored in physiological saline solution containing 0.1% thymol at 4°C [14]. Next were randomly into six experimental groups whitening gel (n=12/group) containing 35% H_2O_2 and NaF and/or TMP. After pigmentation of the enamel disks, bleaching gels were applied once (40 min/session), every 7 days, totaling 3 sessions (21 days). The color alteration was analyzed after pigmentation, 1st, 2nd and 3rd whitening session, and 7 and 14 days after whitening.

2.2. Bleaching Gels Formulation and Experimental Groups

The whitening gels were manufactured by Oralls - Innovation in Oral Health Company (São José dos Campos, SP, Brazil), without pigmentation and packaged in applicator syringes. The base components of the gels were composed of thickener (12% Carbopol), bleaching agent (35% H_2O_2), glycerin and water (q.s.), and NaOH required maintaining pH of approximately 7.0. Depending on the experimental group, 0.05% or 0.1% NaF and 0.25% or 1% TMP was added [9,10]. A market bleaching gel containing 35% H_2O_2 and 2% CaGlu, neutral pH (Whiteness HP Blue, FGM, Joinville, SC, Brazil), was used as a positive control. Thus, six whitening gel experimental were defined: 1) 35% hydrogen peroxide (35% H_2O_2); 2) 35% H_2O_2 + 0.05% NaF (35% H_2O_2/0.05% NaF); 3) 35% H_2O_2 + 0.25% TMP (35% H_2O_2/0.25% TMP); 4) 35% H_2O_2 + 0.05% NaF + 0.25% TMP (35% H_2O_2/0.05% NaF/0.25% TMP); 5) 35% H_2O_2 + 0.1% NaF + 1%

TMP (35% H_2O_2/0.1% NaF/1% TMP) and 6) 35% H_2O_2 + 2% calcium gluconate (35% H_2O_2/2% CaGlu).

2.3. Color Alteration Analysis

2.3.1. Selection and Distribution of Enamel/Dentin Disks

After obtaining the disks, a preliminary initial reading of the color values was carried out, according to the Commissione Internationale de I'Eclairage (CIE) which uses the CIE L*a*b* color system [14,15]. The enamel/dentin disks were fixed on black silicone supports with a diameter of 5.7 mm and a thickness of 3.5 mm standardizing the incidence of the light beam in the Visible Ultraviolet Reflection spectrophotometer (Model UV-2450, Shimadzu, Kyoto, Japan), with wavelength ranging from 400 nm to 700 nm, D65 standard illumination and illumination/observation angle 45/0º. The readings were performed on the enamel face, defining the initial L*a*b* values of the enamel/dentin disks as standard in this experiment. The disks were randomly divided (Excel, Microsoft Corporation, USA) into 6 experimental groups (n=12) with mean values (SD) varying between: L* = 62.72 (2.95) and 65.01 (1.18) (p = 0.170); a* = -1.21 (0.28) and -0.86 (0.26) (p = 0.087); and b* = 5.02 (1.50) and 6.10 (1.24) (p = 0.396).

2.3.2. Pigmentation of Enamel/Dentin Disks

For the pigmentation of the enamel/dentin disks, these were stored in microtubes (Kasvi K6-0150, 1.5 mL, São José dos Pinhais, PR, Brazil) containing 1 mL of black tea infusion at room temperature. The infusion was made using 1.6 g of black tea (Chá Matte Leão, Curitiba, PR, Brazil) for each 100 mL of deionized water [15]. The pigmentation process was monitored for 6 days, and the infusion was changed daily. Next, the color alteration was determined.

2.3.3. Bleaching Gels Treatments and Color Alteration Measurement

After pigmentation, the whitening gels were applied to the enamel surface (0.04 mL), for 40 min, utilizing dosing syringe and microbrush (KG Sorensen, Cotia, SP, Brazil). During 21 days, 3 whitening sessions were performed with an interval of 7 days. The gels were removed with gauze followed by washing with deionized water for 30 s. Amongst whitening sessions, the disks were kept in individual plastic containers containing 2 mL of artificial saliva [14] with the following formulation: 1.5 mmol/L de $Ca(NO_3)_2.H_2O$; 0.9 mmol/L de $NaH_2PO_4.H_2O$; 150 mmol/L de KCl;

in 0.1 mol/L of sodium cacodylate buffer, pH 7.0 [9]. Artificial saliva was renewed every day. The measurement of the colors were performed after the 1st, 2nd and 3rd whitening session, as well as at 7 and 14 days after the whitening was finished. After that, it were calculated the absolute differences (Δ) of the color coordinates (L*, a*, b*) between the time analysis (post-stained, post-bleaching sessions and post 7 and 14-days) and initial values: ΔL* (positive value indicate more light; negative indicate more dark), Δa* (positive indicate more red; negative indicate more green) and Δb* (positive indicate more yellow; negative indicate more blue). To determine the total color alteration among the three coordinates, the following formula was used: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ [14,15].

2.4. Surface and Cross-sectional Hardness Analysis

The enamel/dentin disks had their enamel surface flatted. After polishing, the initial surface hardness (SHi) was performed using Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) with Knoop diamond indenter under 25 g for 10 s. In each enamel/dentin disk, five indentations were made with a distance of 100 μm from each other [9,13]. The enamel/dentin disks were selected by SHi and randomly divided (Excel, Microsoft Corporation, USA) into the 6 previously defined experimental groups (n=12) with mean values (SD) ranging from 363.9 (6.3) to 366.7 (5.6) KHN ($p = 0.642$). Hereafter, the disks were submitted to the three bleaching sessions as previously described. After the 21-day period, the final surface hardness (SHf) was determined to calculate the percentage of loss of surface hardness (%SH = [[SHf–SHi]/SHi] ×100) [10].

After determining of SHf, the enamel/dentin disks were sectioned in the center, and one of the halves were included in acrylic resin and polished as previously described. The cross-sectional hardness was performed using the Micromet 5114 Knoop hardness tester, with a load of 5 g for 10 s. Three sequences of equidistant indentations were performed 100 μm from each other, and at 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 180 μm of the surface. The mean values at all 3 measuring points at each distance from the surface were then averaged. The integrated area below the curve (KHN × μm) using the hardness values was calculated by the trapezoidal rule [9,13].

2.5. Determination of H_2O_2 Trans-Amelodentinal Penetration

The enamel/dentin discs (n=12/group) were attached to upper compartment of the artificial pulp chamber (APC) utilizing silicone rings and sealed with melted pink wax #7 (Wilson®, Polidental,

Cotia, SP, Brazil), based in the study of Trindade et al. [16]. The lower compartments of the APCs, containing the dentin surface, were immersed in 1 mL of sodium acetate buffer solution (2.0 mol/L, pH 4.5 by acetic acid, Sigma Chemical Co, St Louis, MO, USA) placed in 24-wells of cell culture plates (Costar Corp., Cambridge, MA, USA) [17]. In these conditions and in a humid environment at 37ºC, the bleaching gels were once applied as described previously. To determine the amount of H_2O_2 in the buffer solution, the Mottola et al. [18] colorimetric method was used. After 30 minutes of whitening, were added 1 mL of violet leukocrystal (0.5 mg/mL in HCl, Sigma Chemical Co, St Louis, MO, USA), 50 µL of enzyme horseradish peroxidase (1 mg/mL in water, Sigma Chemical Co, St Louis, MO, USA) and 3 mL of deionized water [17]. A standard curve of H_2O_2 concentrations (0.5 to 5.0 µg/mL) in the same conditions of the samples was built and readings were performed utilizing spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at wavelength of 596 nm.

2.6. Evaluation of Trans-Amelodentinal Cytotoxicity

For that, 30,000 cells/cm^2 from immortalized odontoblast-like cell line (MDPC-23) were seeded in each well of sterile 24-well plates (Costar Corp., Cambridge, MA, USA), and maintained in a humidified incubator with 5% CO_2 and 95% air at 37ºC for 72 hours [16]. The ACPs containing enamel/dentin disks (n=5), previously sterilized by ethylene oxide gas [16]. The bleaching gels were applied according to H_2O_2 Trans-Amelodentinal Penetration Test, and after 30 minutes, 1 mL culture medium containing the permeated components from H_2O_2 were added to previously prepared odontoblast-like cell culture.

The analysis of cell viability (n=5) was performed by using enzymatic assay of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma). The cells were incubated for 4 h with MTT solution (Sigma–Aldrich Corp.) at 37oC and 5% CO_2. After that, the absorbance of formazan crystals in the viable cells was assessed in an ELISA microplate reader (570 nm) (Tp Reader, Thermoplate, Nanshan District, Shenzhen, China). The average absorbance of the negative control group was considered to be 100% cell viability, and the percentage of cell viability for the lightened groups calculated based on this parameter [16].

For determination of alkaline phosphatase activity (ALP, Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil), cells were subjected to cell lysis with 150 µL of 0.1% sodium lauryl sulfate (Sigma) during 40 minutes. 50 uL of the lysed cells, 100 µL of thymolphthalein monophosphate (22 mmol/L, pH 10.1) was added in a water bath at 37ºC. After color reagent (94 mmol/L of sodium carbonate in 250 mmol/L of sodium hydroxide) was added reacting with thymolphthalein

hydrolyzed by alkaline phosphatase [19]. The absorbance values for ALP were determined at 570 nm wavelength (Synergy H, Biotek, Winooski, Vermont, USA). ALP values were normalized by total protein measured in the remaining samples adding 100 µL of Lowry reagent solution (Sigma) by 20 minutes and 50 µL of Folin phenol reagent solution (Sigma). After 30 minutes, the absorbance at 655 nm wavelength under a standard curve of protein (13 to 100 µg/mL) was determined. The values of ALP activity were expressed in mg of protein and percentage calculated considering the negative control group as 100% of activity.

The formation of mineralization nodules was determined after fixation of cells in 70% ethanol (4º C for 1 hour), water bath and incubation in Alizarin Red (40 mmol/L, pH 4.2; Sigma) for 15 minutes under stirring. Following three water rinsing, a solution of cetylpyridine chloride (10 mmol/L, phosphate buffer solution-PBS, pH 7.0; Sigma) was applied for 15 minutes to produce nodules dissolution. The absorbance at 570 nm was measured and percentage calculated as described previously.

For analysis of cell morphology, cells were dehydrated in an increasing sequence of ethanol (30%, 50%, 70%, 90% 2x, 100%; 30 minutes each) followed by rinsing 3 times (20 minutes/rinse) with 200 µL of 1,1,1,3,3,3-hexamethyldisilazane (HMDS 98%, ACROS Organics, New Jersey, NY, USA). Then, cover glasses were mounted on metallic stubs, maintained in a vacuum desiccator for 72 h, sputter-coated with a gold layer [19]. The cells morphology was analyzed utilizing a scanning electron microscope (JEOLJMS-T33A Scanning Microscope, Tokyo, Japan) in ×-500 magnification.

2.7. Statistical Analysis

The statistical program Sigmaplot® for Windows version 12.0 was used, with a significance level of 5%. For the color alterations data, the different bleaching gels and the time analysis were considered as variation factors and, as variables, the CIE parameters: L*, a*, b* and ΔE. The data were heterogeneous and were subjected to two-way analysis of variance (ANOVA) of repeated measures followed by the Student-Newman-Keuls multiple comparison test. For hardness analysis, H_2O_2 penetration and cytotoxicity evaluation, the values were considered as variables; and bleaching gels as a variation factor. The values were homogeneous and were submitted to one-way ANOVA followed by the Student-Newman-Keuls test. The data of hardness and H_2O_2 penetration were subject to Pearson's correlation.

3. Results

The process of pigmentation of the enamel sample reduced the luminosity (Figure 1A), increased the red and yellow chromatography (Figure 1B and 1C), with great rise in the total difference of color among the coordinates (Figure 1D) and no difference among the groups. After the 1st bleaching session, the enamel became lighter, less red and bluer. With the 2nd and 3rd sessions bleaching, lighter enamel was established with a low content of red and a higher content of blue, differing the 1st session. This was reflected in the increase in the total color alteration (Figure 1D). Chromatic stability did not differ statistically after 7 and 14 days from last bleaching session, except to 35% H_2O_2/0.05% NaF group.

All bleaching gels led to hardness loss (Table 1) and the greatest loss of hardness was approximately between the depths of 0 and 40 μm into the enamel for all groups tested (Figure 2). Compared to the 35% H_2O_2 group, the addition of NaF reduced the surface hardness loss (SHf: $p<0.001$; %SH: $p<0.001$), the TMP reduced the hardness loss in the inner part of the enamel ($p=0.036$), and CaGlu had the highest losses ($p=0.004$; $p<0.001$; $p<0.001$, respectively). Bleaching gel containing 35% H_2O_2/0.1% NaF/1% TMP led to the lowest values of hardness loss ($p<0.001$; $p<0.001$; $p<0.001$; respectively) followed by 35% H_2O_2/0.05% NaF/0.25% TMP (Table 1). In addition, it can be noted that the hardness values of the 35% H_2O_2/0.1% NaF/1% TMP group were higher in all profiles (Figure 2). The bleaching gel containing CaGlu had the highest values of trans-amelodentinal penetration of H_2O_2 ($p<0.001$) and 35% H_2O_2/0.1% NaF/1% TMP led to the lowest ($p<0.001$). There was negative correlation between trans-amelodentinal penetration of H_2O_2 and SHf (Pearson's $r = -0,682$; $p <0.001$), %SH (Pearson's $r = -0,716$; $p <0.001$) and integrated area (Pearson's $r = -0,676$; $p <0.001$).

All groups reduced cell viability, alkaline phosphatase activity and formation of mineralization nodules compared to negative control group ($p<0.001$). The viability was around 9% (Fig. 3A) except for bleaching gel containing 35% H_2O_2/0.1% NaF/1% TMP with 12.8% ($p<0.05$). Alkaline phosphatase activity (Fig. 3B) was higher for groups containing TMP compared to others whitening gels ($p<0.05$). The formation of mineralization nodules (Fig. 3C) was greater for gels containing NaF/TMP or CaGlu ($p<0.05$). The number of cells adhered to cover glass had a great reduction for the groups containing 35% H_2O_2 compared to the negative control (Fig. 4). This reduction was lower for groups containing NaF/TMP; however, the alterations of cell morphology were intense for all bleaching gels (Fig. 4).

4. Discussion

It is extremely important to maintain the functional properties and clinical effectiveness of a product when adding and/or changing its chemical composition. The addition of NaF/TMP did not alter the bleaching properties of 35% H_2O_2 gel; however, reduced the mineral loss and trans-amelodentinal cytotoxicity. Thus, the null hypothesis was partially rejected. All bleaching gels, regardless the addition of NaF, TMP, CaGlu or association NaF/TMP, maintained the bleaching capacity of H_2O_2 producing clinically relevant results. The increase in the ΔL* values (Fig. 1A) and a decrease in the Δa* and Δb* values (Fig. 1B and 1C) clearly indicates the color change to white and a decrease in the redwish and yellowish hue, producing the whitening effect on the tooth structure [20]. These outcomes are in agreement with the Borges et al. [14] for 35% H_2O_2. For the patients, whitening satisfaction is more related with variations in the b* coordinate than a* and L* [14]. Despite being considered less important in bleaching, in the present study a* component showed great variation, probably due to the pigmentation previously produced. In this case, the reduction in a* values had a great impact on the total color change.

All gels produced the most pronounced changes in the first whitening session. These findings are in agreement with those of Kwon et al. [4], who also observed a marked whitening effect after the first session with 35% H_2O_2 and less chromatic changes in subsequent sessions. According to Sun [21], this is because the darker molecules react more easily with free radicals, causing great visual impact even in the first application of tooth whitening with a highly concentrated agent. As important as the whitening capacity, color sustaining or chromatic stability over time (7 days and 14 days after whitening) was maintained for all whitening gels. According to Bizhang et al. [22], color degradation is minimal and linear over time showing suitable color stability throughout 18-month post-treatment. The color alteration and its maintenance over time is a consequence of a large penetration of H_2O_2 in the enamel prisms and reaction with the chromophore molecules resulting in more reflection and less absorption of light [4].

The presence of remineralizing agents in the bleaching gels as calcium [5] or fluoride [5] or its associations [5] can reduce the penetration of H_2O_2. Reducing the diffusion of peroxide through enamel and dentin can lead to less whitening effect, but clinically noticeable to the naked eye [2,5]. Nonetheless, ΔE values greater than 3 would indicate an acceptable bleaching as long as it is associated with a reduction of b* values and increase of L* values [23]. This was observed for all experimental gels tested after treatment sessions (Fig. 1). Except after pigmentation that, despite the ΔE values being greater than 12, the positive Δb* and negative ΔL* values indicate a darkened enamel. Notwithstanding the reduction in the diffusion of H_2O_2 in the groups with TMP

(Table 1), it was not possible to verify a relationship in the whitening effect (Fig. 1). These reductions were around 14% to 26%; half of the percentage observed in the study by Soares et al. [2], when there was less lightening effect. Unlike previous studies [5,24], the addition of calcium and fluoride did not reduce the penetration of H_2O_2 as well as surface and cross-sectional hardness loss (Table 1). Probably the best results with the addition of fluoride are due to a higher concentration (0.2% to 1.1% NaF) [5,24,25] than the present study (0.05% and 0.1% NaF). For calcium, there are formulations with similar concentrations to the present study (2% CaGlu) that have been shown to reduce mineral loss [26], contrary to the present data and other study [5].

During the diffusion of the bleaching agent into the enamel prisms, the products of the degradation of H_2O_2 (reactive oxygen molecules and hydrogen peroxide anions) not only react with the dark-colored chromophore molecules, but also on the organic component (as proteins, lipids and teeth-staining substances) of the enamel [1]. Removal or degradation of the protein matrix located between the enamel prisms and hydroxyapatite crystallites can reduce the enamel's mechanical properties such as hardness, since they act as “glue” between them [1]. According to previous studies, the changes in the mineral component of the enamel produced by peroxide free radicals would only occur in the outermost part of the enamel [1,6] at 10 µm below the surface [6]. Here, it is noteworthy that all experimental gels had their pH adjusted to 7.0 to avoid enamel demineralization due to the acidity of the whitening gel [3]. Differently, the results of the present study showed a great reduction of the hardness in depth mainly in the region up to 50 µm of the surface (Fig. 2), in agreement with the study by Cavalli et al. [25] that observed hardness loss up to 60 µm depth from the outer enamel surface. This shows that H_2O_2 has not only an oxidizing effect, but also an acid effect [1]. It should be noted that in the present study, unlike previous studies, three 45-minute whitening sessions were performed, which may explain the greater mineral loss. Treatment time and number of sessions have an impact on observing changes in enamel hardness. Thus, the reduction in the values of surface and cross-sectional hardness was not only due to the degradation of the organic component of the enamel, but also the loss of its inorganic one. According to ISO 28399, a reduction of up to 10% in the enamel hardness is acceptable for whitening procedures [5]. Considering this information, only the gel containing 0.1% NaF and 1% TMP shows an acceptable surface hardness loss. We can infer that the gel with 0.05% NaF and 0.25% TMP is in the border area (Table 1). This effect is based on altering the selective permeability and diffusion of positively charged ions of CaF^+ and Ca^{2+} and/or neutral species ($CaHPO_4^0$ and HF^0), and reducing of H+ diffusion in the enamel produced by NaF/TMP association [12]. It is possible to consider that in the innermost part of the enamel (> 80 µm; Fig. 2), the reduction of hardness derives more from the degradation of the protein matrix than the

demineralization of the hydroxyapatite crystals, and the association NaF/TMP minimized these structural changes in the matrix. Taking into account what was said earlier, the addition of remineralizing agents to whitening gels should aim not only to reduce the demineralization of hydroxyapatite, but also to minimize the degradation of the enamel protein component. Nevertheless, the diffusion of H_2O_2 through enamel and dentin is necessary for the bleaching effect.

Albeit there is a positive correlation between mineral loss and diffusion of H_2O_2, cell damage was severe (Fig. 3 and 4). Even the addition of 0.1% NaF e 1% TMP showing less cytotoxicity when compared to the other bleaching gels, cell viability was reduced in 87% (Fig. 3A). Although TMP shows a potential to reduce diffusion of H_2O_2, this effect falls far short of what is needed to prevent cell damage. This indicates a great amelodentinal diffusion of non-degradated H_2O_2, mainly due to long application time and high concentration of H_2O_2, and oxidative cell stress induced by H_2O_2 byproducts [2,7,8]. Perhaps concentrations of TMP above 3% and NaF above 0.2% or the use of nanosized TMP might further enhance the protective potential of the bleaching gels. This is because TMP is adsorbed, however, it does not penetrate into the enamel, binds to the OH^- groups on the hydroxyapatite [11] form a TMP-Ca^{2+}-PO_4^- and/or TMP-Ca^{2+}-F layer on the enamel [12]. Based on this, TMP layer might bind to the anions (HO_2^-, OH^-) and cations (H^+) derived from H_2O_2 degradation which explains the effect on enamel demineralization up to 50 µm but insufficient to prevent alterations in cell morphology and in cell death (Fig. 4). In addition to cell viability, the cell regenerative potential was evaluated by the expression of two indicators of odontogenic differentiation: ALP activity and deposition of mineralization nodules [7]. With the addition of TMP to bleaching gels containing 35% H_2O_2, the expression of these markers was greater, regardless of fluoride concentration, when compared to bleaching without TMP (Fig. 3B and 3C). This result was close to that found with a 10% H_2O_2 when applied for 40 minutes [7], however it is related to the cells that remained viable (Fig. 3A e Fig. 4). As previously described, the regenerative capacity observed with the presence of TMP might be related to lower diffusion of H_2O_2; another possibility would have been a slower penetration in these groups and lower quantity of H_2O_2 byproducts (relative oxygen species) [2,8] retained by TMP. Thus, the scientific evidence obtained in the present investigation for the gel with the associations of TMP and NaF can be considered promising. The bleaching gels evaluated are presented as interesting alternatives to be tested in future clinical research, which may result in the development of aesthetically viable office whitening therapies that are biologically compatible with the dentin-pulp complex, so as to effectively reduce side effects teeth whitening increased the safety of the procedure.

5. Conclusion

Taking into account the data obtained and the limitations of the experimental model, we can conclude that: (1) the addition of TMP and NaF in the composition of the bleaching gel to 35% hydrogen peroxide significantly reduces the loss of surface and cross-sectional hardness on the enamel; (2) the addition of NaF/TMP to the bleaching gel does not interfere in the bleaching effectiveness; (3) the presence of TMP in the 35% H_2O_2 gels reduced the penetration trans-amelodentinal; (4) the cell viability and the regenerative capacity were higher, mainly with 0.1% NaF and 1% TMP gel.

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List of Tables

List of figures

Table 1 – Means values (SD) of final surface hardness (SHf), percentage of loss surface hardness (%SH), integrated area (KHN × µm) and trans-Amelodentinal penetration of H_2O_2, according experimental gels (n=12)

Bleaching gels	**Variables**			
	SHf	%SH	KHN × µm	H_2O_2 (µg/mL)
35% H_2O_2	294.0[a] (5.1)	-19.7[a] (1.0)	55,035.8[a] (2,678.7)	8.83[a] (0.64)
35% H_2O_2/ 0.05% NaF	306.3[b] (2.1)	-16.1[b] (0.9)	53,852.3[a,b] (1,216.9)	8.83[a] (0.32)
35% H_2O_2/ 0.25% TMP	296.0[a] (4.4)	-18.6[a] (1.3)	53,223.4[b] (950.4)	7.57[b] (0.56)
35% H_2O_2/0.05% NaF/0.25% TMP	324.6[c] (4.7)	-11.8[c] (1.1)	57,928.2[c] (1,895.5)	7.91[b] (0.54)
35% H_2O_2/0.1% NaF/1% TMP	339.0[d] (9.1)	-7.1[d] (2.1)	61,400.5[d] (1,180.8)	6.52[c] (0.73)
35% H_2O_2/ 2% CaGlu	287.6[e] (3.1)	-21.6[e] (1.1)	51,455.5[e] (1,979.2)	10.01[d] (0.26)

Distinct superscript lowercase letters indicate statistical difference among bleaching gels in each variable (Student–Newman–Keuls test, $p < 0.001$).

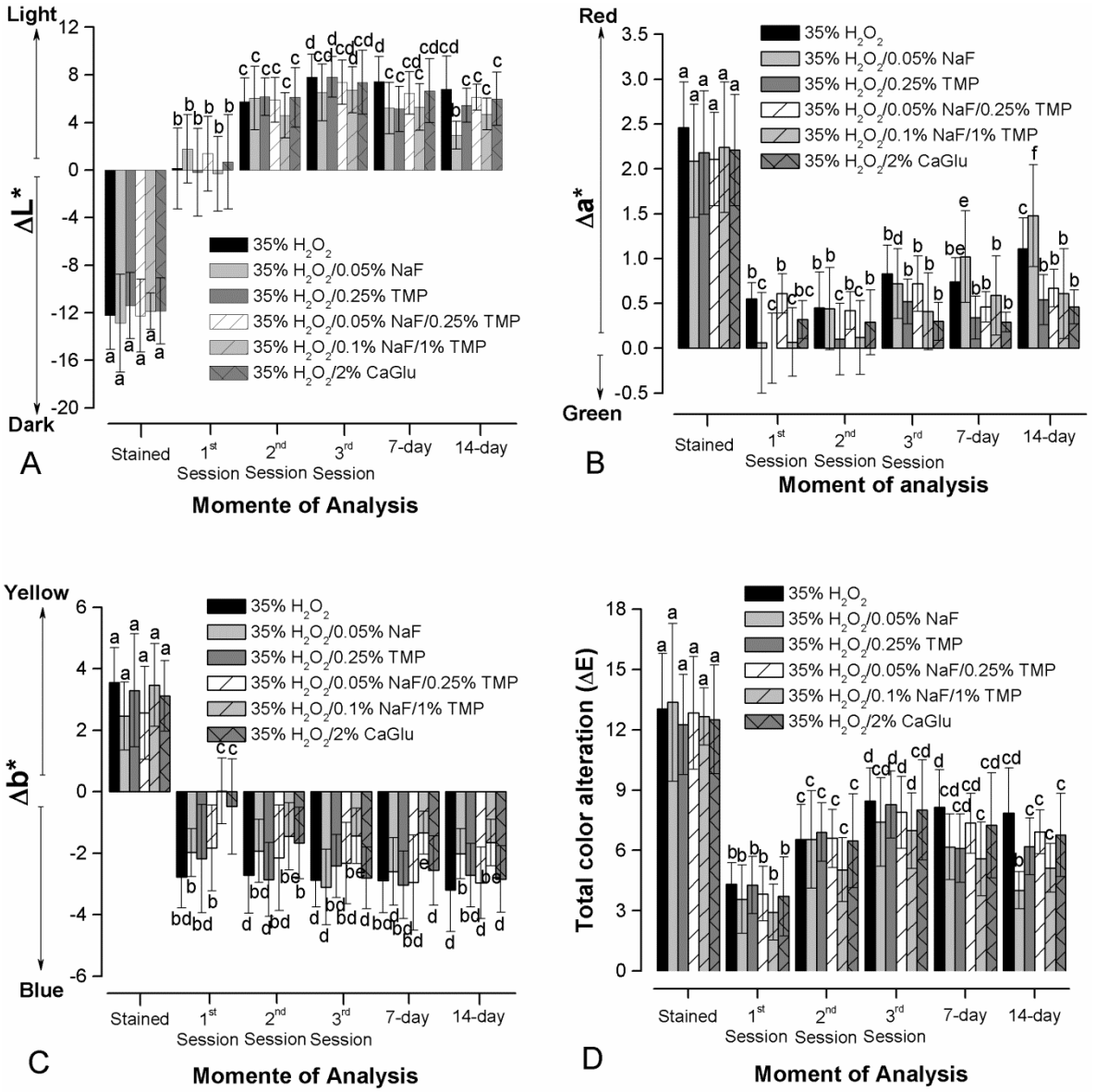


Figure 1 – Means values (SD) of the alteration of: (A) lightness degree (ΔL*), (B) green/red degree (Δa*), (C) blue/yellow degree (Δb*) and total color (ΔE) by Comission Internacionale de I'Eclairage (CIE) accordint to bleaching gels and moment of analysis (n=12). Equal lowercase letters indicate that there is no difference among the bleaching gels at each moment of analysis and among the moments of analysis for each bleaching gel (Student-Newman-Keuls; $p < 0.05$).

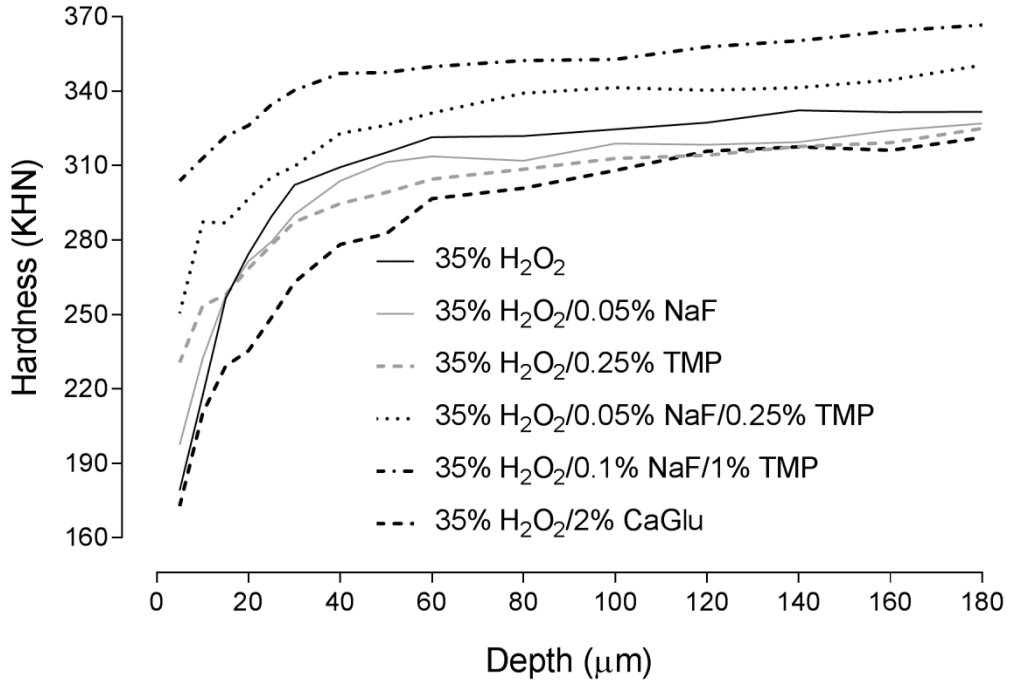


Figure 2 – Cross-sectional hardness profiles at different depths in the enamel (n=12) according to the bleaching gels.

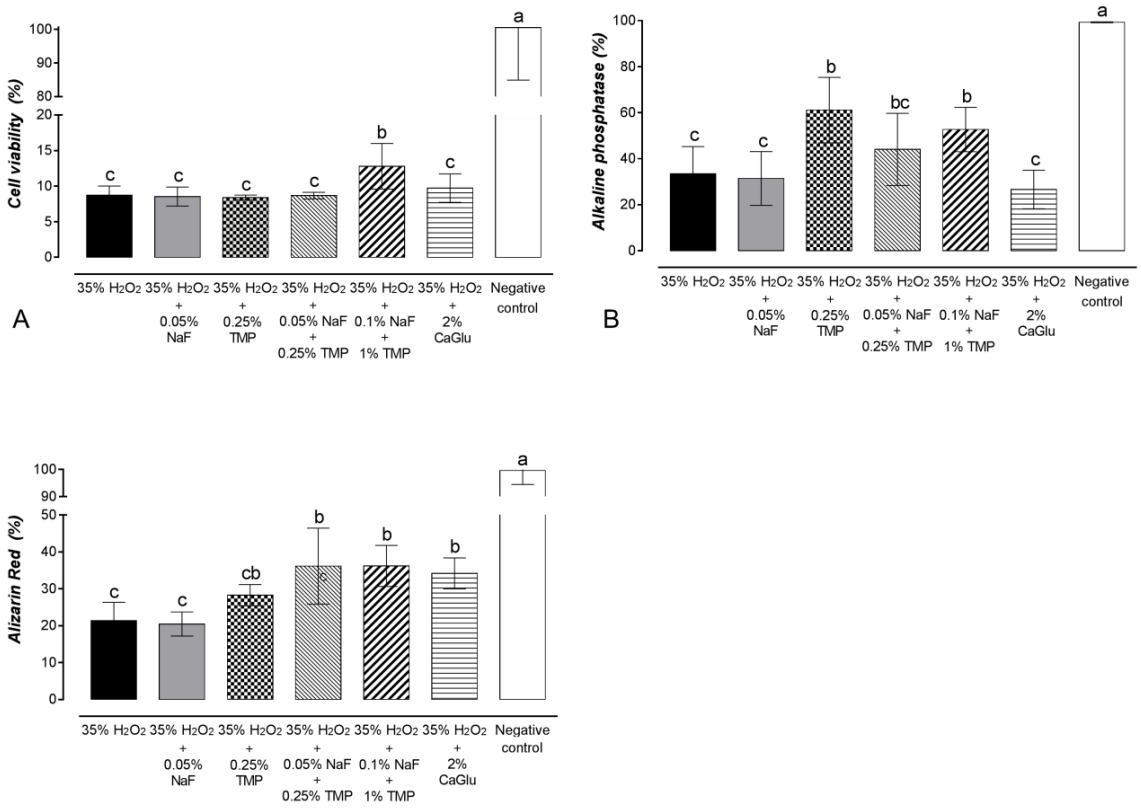


Figure 3 – Graphics representatives of percentage of the Cell viability (A), alkaline phosphatase activity (B) and formation of mineralization nodules (C). Different lowercase letters indicate statistical difference among groups (Student-Newman-Keuls; $p < 0.05$).

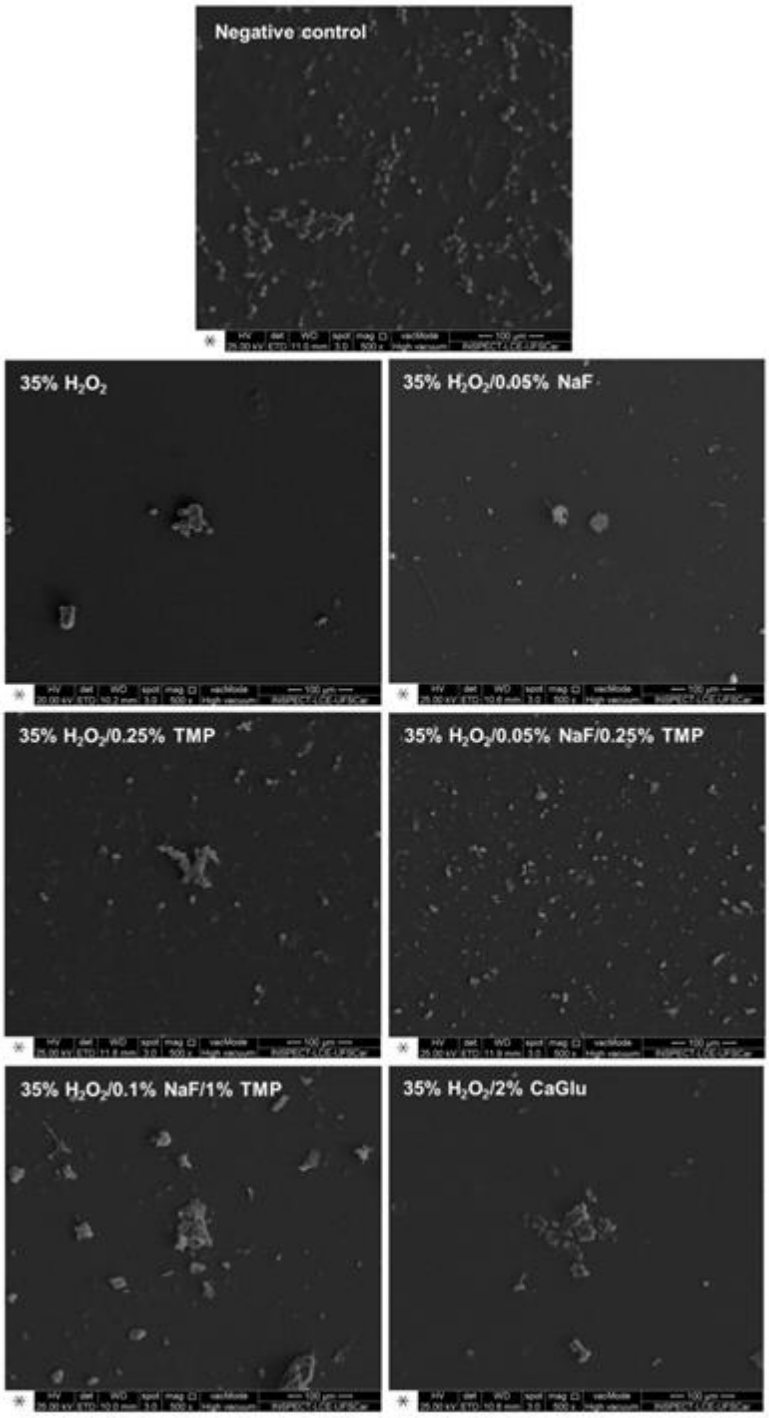
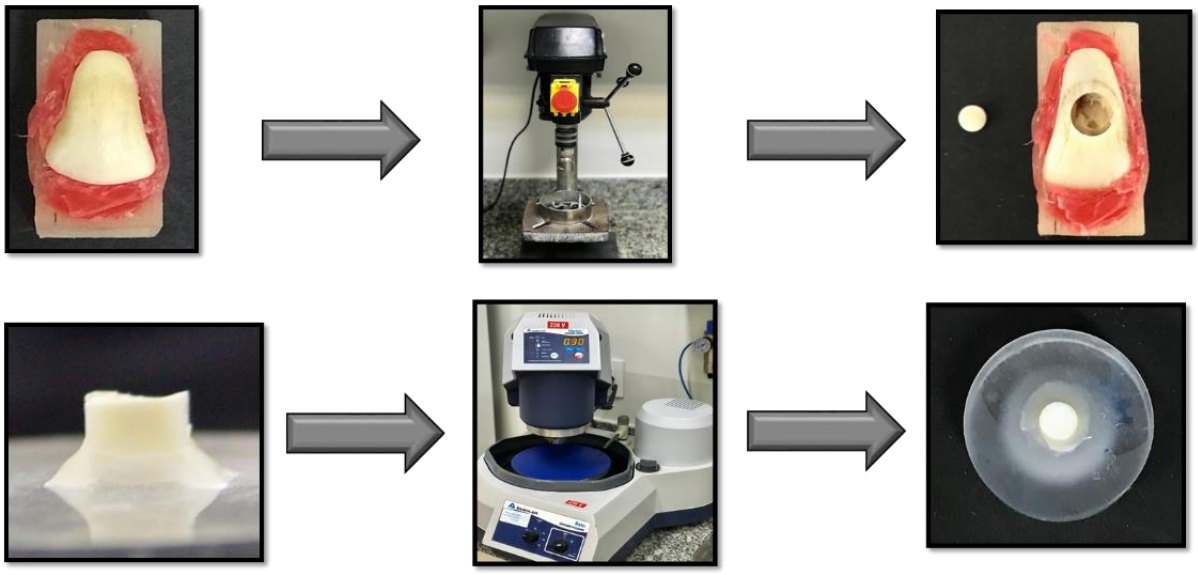


Figure 4 – Photomicrographs from scanning electron microscope (×500) representative of the negative control and bleached groups.

Anexos

ANEXO A

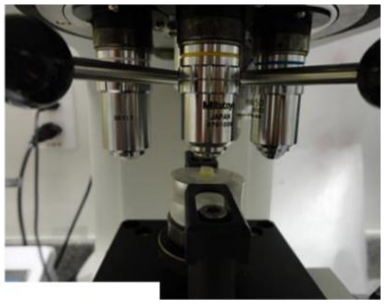
PREPARO DOS DISCOS DE ESMALTE



ANEXO B

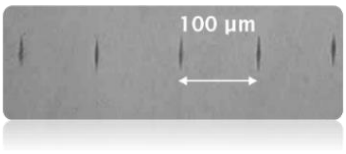
Dureza de Superfície Inicial (SH) e Tratamento com os gés clareadores

Microdurômetro Buehler



Carga de 25 gramas
Tempo 10 segundos

320-380 KHN

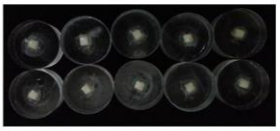


Tratamento com os gés clareadores

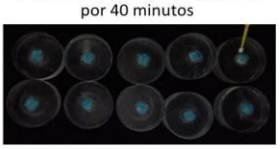
Géis experimentais

HP Blue 35%

Tratamento com Géis experimentais por 40 minutos



Tratamento com Gel HP Blue 35% por 40 minutos



ANEXO C

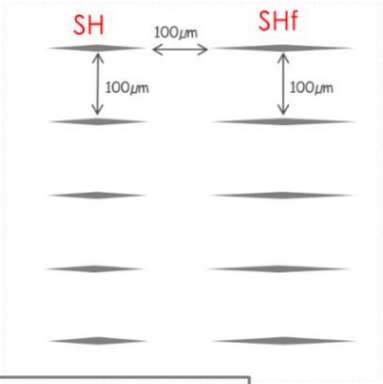
Dureza de Superfície Final e Perda Integrada

de Dureza de Subsuperfície (KHN)

Microdurômetro Buehler

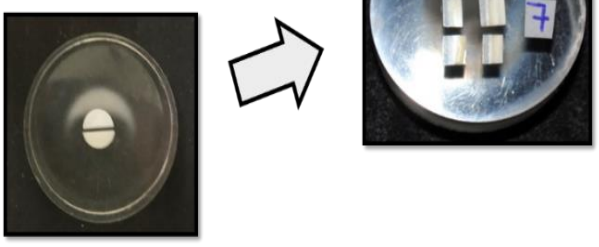
Carga de 25 gramas

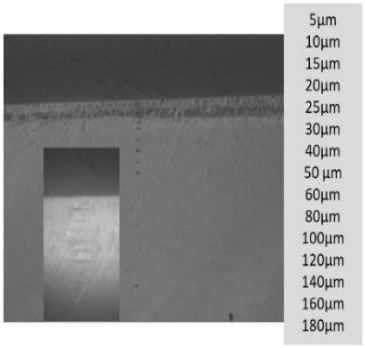
Tempo 10 segundos



Cálculo da porcentagem de perda de dureza de superfície [%SH = ((SHF– SHI)/SHI x 100)].

Perda Integrada de Dureza de Subsuperfície (KHN)





Microduromêtro Buehler

Carga 5g - Tempo 10 seg.

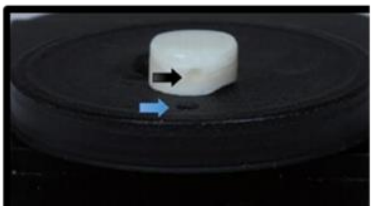
ANEXO D

Análise da Alteração de cor

1 – Profilaxia com pedra-pomes

3 - Espectrofotômetro – Leitura Inicial

2 - Marcação para padronização das leituras



4 – Manchamento solução de Chá - Preto

2g de chá /100mL de água fervente e 100mL por disco; 6 dias a 37°C

Armazenamento em água durante 7 dias/37°C

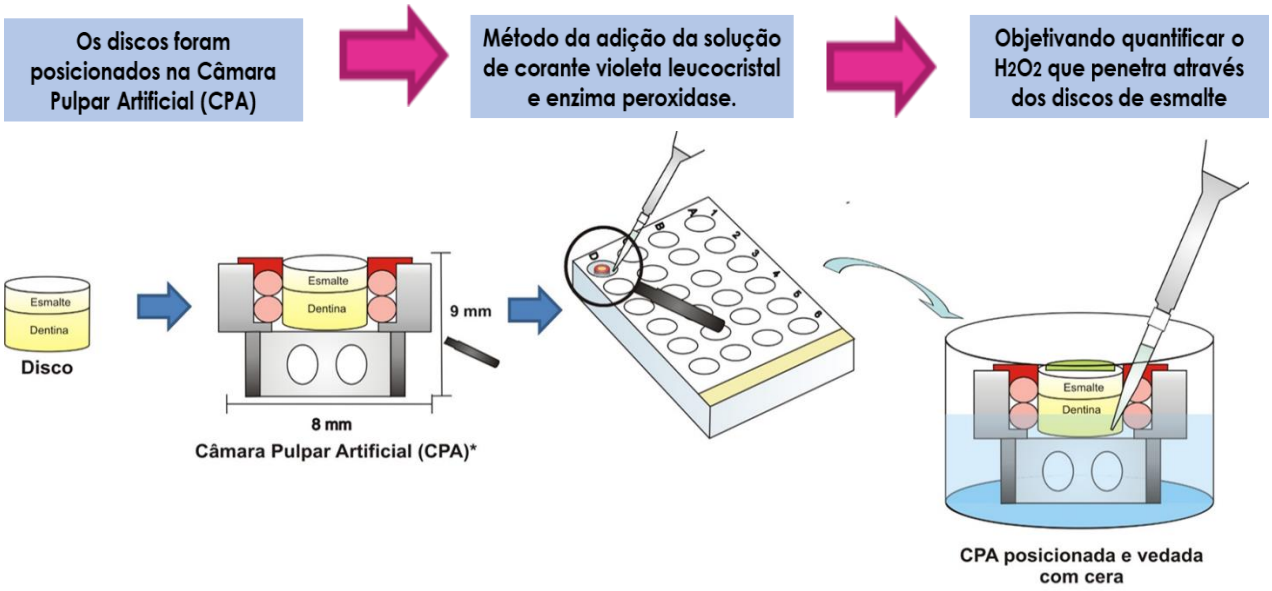
Armazenamento em ambiente úmido com esmalte em contato com a saliva artificial a 37°C por 24h

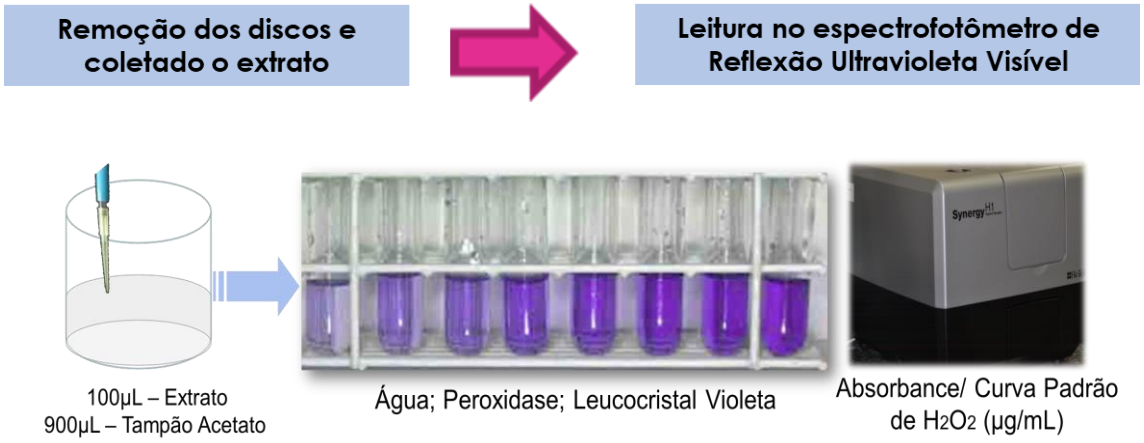
5 – Tratamentos: 3 sessões clareadoras

- ✓ **Leitura pós sessão;**
- ✓ **Leitura pós 7 e 14 dias após o tratamento: estabilização cromática;**

ANEXO E

Penetração trans-amelodentinária

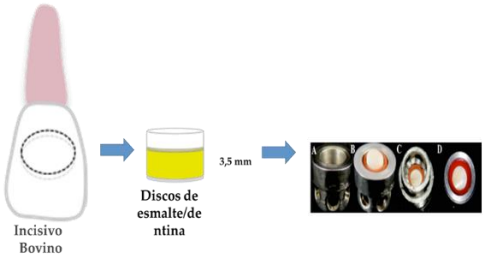




ANEXO F

Citotoxicidade trans-amelodentinária

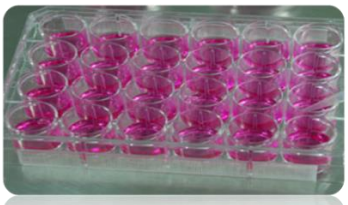
Obtenção dos Espécimes



Cultura Celular

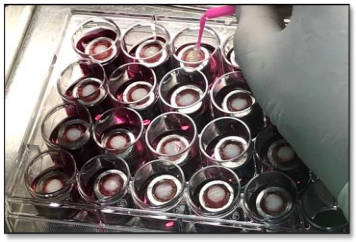
Células de Linhagem Imortalizada de Células Odontoblastóides MDPC - 23

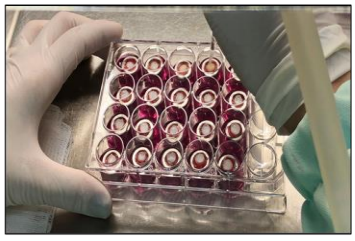
Células cultivadas e subcultivadas em garrafas de 75cm²



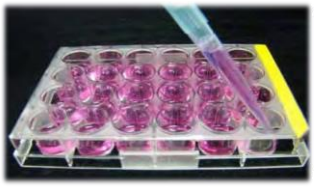
Células semeadas em padrão com 80% de confluência (60.000 cels/well por 24 h)

Incubação 24h

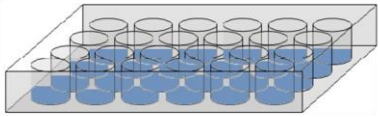




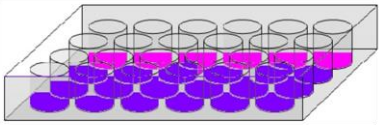
Após o clareamento, CPAs foram removidas



O extrato foi aplicado por 1h nas células



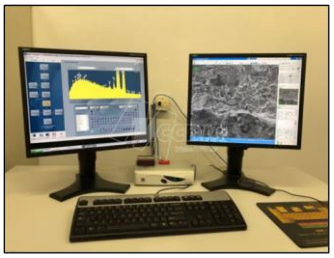
4 h



Realização do teste do MTT

A porcentagem de viabilidade celular foi calculada considerando G1 como 100% de viabilidade celular

Fluorescência



- ✓ Atividade de Fosfatase Alcalina (ALP)
- ✓ Deposição de Nódulos de Mineralização
- ✓ Morfologia Celular pela Microscopia Eletrônica de Varredura (MEV)