UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

MATHEUS KURY RODRIGUES

TERAPIAS CLAREADORAS ALTERNATIVAS UTILIZANDO CONCENTRAÇÕES REDUZIDAS DE GÉIS DE PERÓXIDO, LED VIOLETA E/OU NANOPARTÍCULAS DE DIÓXIDO DE TITÂNIO CO-DOPADAS COM NITROGÊNIO E FLÚOR

ALTERNATIVE BLEACHING THERAPIES USING LOWER CONCENTRATIONS OF PEROXIDE-BASED GELS, VIOLET LED, AND/OR TITANIUM DIOXIDE NANOPARTICLES CO-DOPED WITH NITROGEN AND FLUORINE

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Clínica Odontológica, na Área de concentração em Dentística.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Clinics, in Operative Dentistry Area.

Orientadora: Prof.ª Dr.ª Vanessa Cavalli Gobbo

Este exemplar corresponde à versão final da tese defendida pelo aluno Matheus Kury Rodrigues e orientada pela Profª Drª Vanessa Cavalli Gobbo.

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A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 01 de dezembro de 2023, considerou o candidato MATHEUS KURY RODRIGUES aprovado.

PROFª. DRª. VANESSA CAVALLI GOBBO

PROFª. DRª. JOSIMERI HEBLING COSTA

PROFª. DRª. SANDRINE BITTENCOURT BERGER

PROFª. DRª. GISELLE MARIA MARCHI

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

“Ninguém quer saber o que fomos, o que possuíamos, que cargo ocupávamos no mundo; o que conta é a luz que cada um já tenha conseguido fazer brilhar em si mesmo.”

(Chico Xavier)

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RESUMO

Esta tese avaliou a eficácia e efeitos à estrutura dental de géis clareadores irradiados com LED violeta (LED) e/ou incorporados com nanopartículas de dióxido de titânio co-dopadas com nitrogênio e fluoreto (TiO_2). No *Artigo 1*, foram avaliadas estabilidade de irradiância, homogeneidade do feixe e transmissão de luz do LED e alteração de cor (ΔE_{00}, ΔL, Δa, Δb) de blocos (esmalte-dentina) pigmentados ou não com chá preto ou fumaça de cigarro previamente ao clareamento com LED ou gel comercial de peróxido de hidrogênio (HP) 40%. No *Artigo 2,* alterações de cor (ΔE_{ab}, ΔE_{00}, ΔSGU) e de índice de clareamento (ΔWI_D) de 100 voluntários de um ensaio clínico randomizado-controlado foram calculadas 6 e 12 meses após clareamento com LED, peróxido de carbamida (CP) 37%, LED/CP, HP 35% ou LED/HP. No *Artigo 3,* os blocos foram clareados com géis experimentais contendo HP (6, 15 ou 35%), incorporados com TiO_2 (0, 5 ou 10%) e irradiados (LED). ΔE_{00}, ΔWI_D, pH dos géis, proporção mineral [carbonato(CO_3^2)/fosfato(PO_4^3)], topografia de superfície, metabolismo e composição de biofilme *(S Mutans)* sob esmalte clareado foram avaliados. No *Artigo 4,* microdureza de superfície (KNH) e transversal (ΔS), profundidade de lesão (luz polarizada), rugosidade (Ra), morfologia de superfície e proporção mineral [cálcio(Ca)/fósforo(P)] do esmalte foram avaliados mediante os mesmos protocolos. No *Artigo 5,* HP 6% e 35% com 5% TiO_2 e LED foram aplicados em discos (esmalte-dentina) acoplados a câmaras pulpares artificiais. Foram avaliados ΔE_{00}, ΔWI_D, difusão de HP, viabilidade (%MDCP-23), estresse oxidativo (EOx), morfologia e fluorescência (Live/Dead) de células de linhagem odontoblásticas. Todos os testes quantitativos foram submetidos à análise estatística, respeitando a normalidade e homoscedasticidade (α=5%). O feixe do LED é heterogêneo, 98% da luz violeta foi absorvida por 1mm de esmalte e a irradiância não foi constante, sendo significativamente afetada pela distância (8mm), região da ponteira e tempo total. Não houve diferença ($p>0,05$) entre LED e HP 40% para ΔE_{00}, ΔL, Δa e Δb apenas para fumaça de cigarro. Após 6 e 12 meses de acompanhamento clínico, clareamento com LED exibiu os menores valores de ΔE_{ab}, ΔE_{00}, ΔSGU e ΔWI_D, e LED não aumentou ΔWI_D promovido por CP e HP ($p>0,05$). Entretanto, LED/CP não exibiu diferença com HP ($p>0,05$) e LED elevou ΔE_{00} de CP e HP ($p<0,05$). 6%HP+5%TiO_2+LED igualou as médias de ΔE_{00} e ΔWI_D de HP35%. TiO_2 elevou levemente o pH dos géis e estes não alteraram a topografia do esmalte, mas todos os protocolos executados reduziram ($p<0,05$) CO_3^2/PO_4^3 e não impediram formação de biofilme. A aplicação dos géis contendo nanopartículas não alterou significativamente KHN, ΔS, Ra, Ca/P e morfologia de superfície comparado ao esmalte não-clareado, independentemente de LED. TiO_2 e LED diminuíram a difusão de peróxido e EOx, aumentaram a %MDCP-23 e preservaram a morfologia celular. 6%HP+5%TiO_2+LED promoveram a menor citotoxicidade. Sendo assim, LED violeta sozinho não apresenta desempenho clínico satisfatório e sua estabilidade operacional é questionável; contudo, poderia aumentar a eficácia de géis clareadores menos concentrados, principalmente quando incorporados com nanopartículas co-dopadas de TiO_2, oferecendo protocolos seguros às propriedades físicas e biológicas da estrutura dental.

Palavras-chave: Clareamento Dental, Peróxido de Hidrogênio, Diodos Emissores de Luz, Dióxido de Titânio.

ABSTRACT

This thesis evaluated the efficacy and effects on tooth structure caused by bleaching gels irradiated with violet LED (LED) and/or incorporated with titanium dioxide nanoparticles co-doped with nitrogen and fluorine (TiO_2). The *Article 1* evaluated the LED's irradiance stability, light beam homogeneity, violet light transmission, and color change (ΔE_{00}, ΔL, Δa, Δb) of blocks (enamel-dentin) stained or not with black tea and cigarette smoke after bleaching with LED or commercial 40% hydrogen peroxide (HP) gel. In *Article 2*, color (ΔE_{ab}, ΔE_{00}, ΔSGU) and whiteness (ΔWI_D) changes of 100 volunteers from a randomized-controlled clinical trial were calculated 6 and 12 months after bleaching with LED, 37% carbamide peroxide (CP), LED/CP, 35% HP, LED/HP. In *Article 3*, blocks were bleached with experimental gels containing HP (6, 15 ou 35%) incorporated with TiO_2 (0, 5 ou 10%) and LED-irradiated. ΔE_{00}, ΔWI_D, gel's pH, mineral ratio, [carbonate(CO_3^{2})/phosphate(PO_4^{3})], surface topography, *S Mutans* biofilm's metabolism and composition were tested. In *Article 4*, surface (KHN) and cross-sectional (ΔS) microhardness, lesion depth (polarized light), surface roughness (Ra) and morphology, and mineral ratio [calcium(Ca)/phosphorous(P)] were evaluated. In *Article 5*, 6% and 35% HP with 5% TiO_2 and LED were applied on disks (enamel-dentin) coupled with artificial pulp chambers. This study tested ΔE_{00}, ΔWI_D, HP diffusion, viability (%MDCP-23), oxidative stress (EOx), and odontoblast-like cells' morphology and fluorescence (Live/Dead). All the quantitative tests were submitted to statistical analyses, respecting the normality and homoscedasticity (α=5%). The LED's light beam is heterogeneous, 98% from the light was absorbed by 1-mm thick enamel, and the irradiance was not stable, being significantly affected by the distance (8mm), region and time. No difference ($p>0.05$) was detected between LED and 40%HP in terms of ΔE_{00}, ΔL, Δa and Δb only for cigarette smoke. After 6 and 12 months of a clinical follow-up, bleaching with LED exhibited the lowest ΔE_{ab}, ΔE_{00}, ΔSGU and ΔWI_D values, and LED did not increase the ΔWI_D promoted by CP and HP ($p>0.05$). However, LED/CP did not present difference with HP ($p>0.05$) and LED enhanced ΔE_{00} for CP and HP ($p>0.05$). 6%HP+5%TiO_2+LED exhibited ΔE_{00} and ΔWI_D similar to HP35%. TiO_2 slightly increased the gel's pH, which did not alter the enamel's topography, but all the protocols reduced ($p<0.05$) CO_3^{2}/PO_4^{3} and did not impede the biofilm formation. TiO_2-containing gels did not significantly affect KHN, ΔS, Ra, Ca/P and morphology compared to non-bleached enamel, independently of LED. TiO_2 and LED reduced the HP diffusion and EOx, increased the %MDCP-23, and preserved the cell morphology. 6%HP+5%TiO_2+LED promoted the lowest citotoxicity. Therefore, violet LED alone does not present a satisfactory clinical behavior and its operational stability is questionable. On the other hand, it could enhance bleaching gel's efficacy, especially low-concentrated gels incorporated with co-doped titanium dioxide nanoparticles, protocols safe to the physical and biological properties of tooth structure.

Key words: Tooth Bleaching, Hydrogen Peroxide, Light-emitting Diodes, Titanium Dioxide.

LISTA DE ABREVIATURAS E SIGLAS

% – Porcento / Percent

~ – Aproximadamente / Approximately

≅ – Aproximadamente / Approximately

λ – Comprimento de onda / Wavelength

⌀ – Diâmetro / Diameter

ºC – Graus centígrados / Degrees centrigrades

Δa – a* change

Δb – b* change

ΔE_{00} – Color change (CIEDE2000)

ΔE_{ab} – Color change (CIELAB1973)

ΔL – Luminosity change

ΔSGU – Subjective color change (Artigo 2)

ΔWI_D – Whiteness index for dentistry change

μg – Microgramas/ Micrograms

μm – Micrometro / Micrometter

μL – Microlitro / Microlitter

3-D – Three-dimensional

a* – Coordinate a* (red + / green -)

AFM – Microscopia de Força Atômica / Atomic Force Microscopy

APC – Artificial pulpar chamber

ATR – Attenuated total reflectance

b* – Coordinate b* (yellow + / blue -)

BMW – Bright Max Whitening (MMOptics)

BT – Black tea (Artigo 1)

C – Chroma

CA – Calcein AM

Ca – Cálcio / Calcium

$Ca_{10}(PO_4)_6(OH)_2$ – Hydroxyapatite

$CaCl_2$ –Calcium chloride

CFU – Colony-forming unit

C_2H_5OH – Ethanol

$C_{18}H_{34}O_2$ – Oleic acid

$C_{18}H_{35}NH_2$ - Oleylamine

cm – Centímetro / Centimeters

CO_2 – Carbon dioxide

CO_3^2 – Carbonato / Carbonate

COM – Comercial / Commercial

CONT – Control - without staining (Artigo 1)

CP – Peróxido de Carbamida / Carbamide Peroxide

CS – Cigarette Smoke (Artigo 1)

DH – Dentin hypersensitivity

EDS – Espectroscopia de energia dispersiva por raio-X / Energy-dispersive X-ray spectroscopy

EPS – Extracellular polymeric substances

EthD-1 – Ethyl1 homodimer-1

FTIR – Fourier transform infrared spectrometer

g – Gramas/ Grams

H – Hue

H_2O_2 – Hydrogen Peroxide

HMDS - 1,1,1,3,3,3-hexamethyldisilazane

$HO_2^{\bullet}$ – Perhydroxyl radical

HP – Peróxido de Hidrogênio / Hydrogen Peroxide

IPB - In-office power bleaching (Artigo 3)

KCl – Potassium chloride

KHN – Knoop Hardness Number

L* – Luminosity (black - / white +)

LED – Luz Emissora de Diodo / Light-emitting Diode

LED – Violet LED light (Artigo 2)

LT – Left upper central incisor (Artigo 1)

LT – Violet light irradiation (Artigos 3, 4 e 5)

MDPC-23 – Células de linhagem odontoblástica imortalizada / Immortalized odontoblast-like cells

mg – Miligramas / Miligrams

Milli-Q – Água ultrapurificada / Ultrapure water

min – Minuto / Minute

mL – Mililitros / Milliliters

Mm – Milímetro / Millilitter

mm – Milímetros / Millimeters

mM – Milimolar / Millimolar

mW/cm^2 – miliWatts per centimeter square (Artigo 1)

N_TiO_2 – Nitrogen-doped titanium dioxide

NAg_TiO_2 – Nitrogen- and silver-doped titanium dioxide

NaH_2PO_4 –Sodium phosphate

NC – Negative control (Artigos 4 e 5)

NF_TiO_2 – Nitrogen- and fluorine-doped titanium dioxide

NH_4F – Ammonium fluoride

nm – Nanômetros / Nanometers

NP – Nitrogen- and fluorine-doped titanium dioxide nanoparticles

$OH^{\bullet}$ – Hydroxyl radical

$OOH^{\bullet}$ – Hydroperoxyl radical

P – Fósforo / Phosphorous

PBS – Phosphate-buffered saline

PLM – Microscopia de luz polarizada / Polarized Light Microscopy

PO_4^{3} – Fosfato / Phosphate

psi –Pounds per square inch

Ra – Roughness average

REBEC – Registro Brasileiro de Ensaios Clínicos National Clinical Trials Registry (Artigo 2)

RLU – Relative light unit

ROS – Espécies Reativas de Oxigênio / Reactive Oxygen Species

Rpm –Rotations per minute

Rq – Root mean square roughness

RT – Right upper central incisor (Artigo 1)

s – Segundos / Seconds

SEM – Microscopia eletrônica de varredura / Scanning electron microspopy

T – Tempo / Time

THY - Liquid culture medium

Ti – Titânio / Titanium

$Ti(OBu)_4$ – Titanium(IV) butoxide

TiO_2 – Dióxido de Titânio / Titanium Dioxide

UV – Ultraviolet

UVA – Ultraviolet

V – Volts

v – Volume / Vollum

V-LED – Violet LED light (Artigo 1)

w – Peso / Weight

W/cm^2 – Watts per centimeter square (Artigo 3)

WI_D – Whiteness index for dentistry

SUMÁRIO

1. INTRODUÇÃO

Figurando como um dos procedimentos estéticos mais conservadores e populares entre os pacientes odontológicos, o clareamento dental é capaz de tornar a estrutura dental mais clara por meio de um processo de oxi-redução (Silva et al., 2018, Rodriguez-Martinez et al., 2019). Os peróxidos, em apresentação de gel, são aplicados na superfície do esmalte, difundindo-se até o tecido dentinário através dos espaços interprismáticos do esmalte (Kwon & Wertz, 2015). Hipotetiza-se que as espécies reativas de oxigênio (ROS), resultantes da decomposição do peróxido de hidrogênio, oxidariam as moléculas orgânicas de longas cadeias responsáveis por pigmentarem a dentina, denominadas de cromóforos. Como consequência, tais moléculas intrínsecas seriam eliminadas da estrutura dental ou teriam o comprimento reduzido, tornando a dentina, cuja cor é vista através da translucidez do esmalte, menos pigmentada naturalmente (Kwon & Wertz, 2015, Rodriguez-Martinez et al., 2019).

A terapia clareadora, quando supervisionada pelo cirurgião-dentista, pode ser executada em duas modalidades distintas: caseira ou em consultório (Basting et al. 2012, Joiner & Luo, 2017). A primeira envolve a utilização de géis de peróxido de carbamida (CP) ou de hidrogênio (HP) em baixas concentrações (CP: 10 a 22%, HP: 3,5 a 10%), que são aplicados diariamente pelos próprios pacientes em moldeiras de acetato personalizadas ou pré-fabricadas (Mailart et al., 2023, de Paula et al., 2023). Os géis de CP diferenciam-se dos de HP devido à composição, uma vez que o peróxido de carbamida é combinado com ureia que, quando em contato com água, são quimicamente convertidos em HP e amônia. Estima-se que géis de CP apresentam 1/3 da concentração de HP encontrada em géis de peróxido de hidrogênio (Kwon & Wertz, 2015). São inúmeros os protocolos de clareamento caseiro encontrados na literatura, devido à grande disponibilidade de marcas comerciais e concentrações de CP e HP, o que interfere diretamente no tempo de aplicação diária (30 minutos a 8 horas) e duração da terapia (até 4 semanas) (Luque-Martinez et al., 2016).

O clareamento em consultório, por sua vez, utiliza géis em altas concentrações de HP (35-40%) ou CP (35-37%), porém, é realizado pelo cirurgião-dentista em até 4 sessões com intervalos de 7 dias (Peixoto et al., 2018, Marski et al., 2022). Em comparação à terapia caseira, o clareamento em consultório possibilita o tratamento em

pacientes não-colaborativos e impossibilitados de usarem a moldeira personalizada (Ferretti et al., 2021). Além disso, embora o resultado estético final de ambas as modalidades seja similar (de Geus et al., 2016), a utilização de altas concentrações de HP possibilitaria um alcance mais imediato das alterações de cor e de índice de clareamento (Klaric Sever et al., 2018).

Contudo, há inúmeros relatos na literatura de que géis em altas concentrações de HP causaram alterações significativas e indesejadas na microdureza de superfície (Andrade et al., 2021, Melo et al., 2022) e transversal (Cavalli et al., 2011, Nunes Jr. et al., 2022), rugosidade de superfície (Sa et al., 2013, Wijetenunga et al., 2021), conteúdo mineral (Kury et al., 2020a, Pinto et al., 2019) e resistências intrínseca (Cavalli et al., 2004, Silva et al., 2005, Tam et al., 2013) e de união (Feiz et al., 2017, Olmedo et al., 2021) do esmalte clareado. Ademais, a difusão de HP em direção a câmaras pulpares artificiais foi capaz de afetar negativamente a viabilidade, morfologia e estresse oxidativo de células de linhagem odontoblásticas em uma relação proporcional com a concentração dos géis de HP (Soares et al., 2014, de Almeida et al., 2015, de Oliveira Duque et al., 2017). De um ponto de vista clínico, este cenário pode ser responsável pela ocorrência da sensibilidade dental e efeitos em longo prazo na estrutura pulpar são desconhecidos. De fato, uma análise multi-regressiva e logística indicou que o risco de sensibilidade dental foi 120% maior e a sua intensidade quatro vezes acima para a terapia clareadora em consultório em comparação à caseira, que utiliza menores concentrações de peróxidos (Rezende et al., 2016).

Visando atenuar ou eliminar tais limitações, diversas modificações em protocolos e géis clareadores foram estudadas até o presente momento. Por exemplo, a incorporação de íons cálcio ou fluoreto em géis clareadores apresentou resultados promissores na manutenção das propriedades físicas da superfície do esmalte dental (Cavalli et al., 2018, Torres et al., 2019, Vieira et al., 2020). Ainda, foram propostos protocolos de clareamento em consultório utilizando géis de HP em menores concentrações (6-25%) (Ferraz et al., 2019, Estay et al., 2020). Embora a sensibilidade dental em decorrência da terapia em consultório seja atenuada nestes casos, uma revisão sistemática e meta-análise recente mostrou baixa certeza de evidência de que a eficácia clareadora seja mantida em consultório quando há diminuição da concentração

de HP (Maran et al., 2020). Outras revisões sistemáticas também mostraram que o uso de fontes luminosas (luz halógena, arco de plasma, laser de argônio, luz emissora de diodo azul, entre outros) não são capazes de aumentar significativamente a eficácia de géis clareadores (He et al., 2012, Maran et al., 2018, Maran et al., 2019), o que poderia ser um fator adjuvante na melhoria da eficácia destes protocolos utilizando géis menos concentrados.

Neste contexto, uma nova geração de luz emissora de diodo (LED) em comprimento de onda violeta foi introduzida no mercado odontológico para clareamento em consultório, com a proposta de não apenas irradiar géis de HP (35-40%), mas também de ser combinada com CP (35-37%) ou utilizada sem a presença de géis clareadores (Zanin, 2016, Lago et al., 2017, Rastelli et al., 2018). O fabricante inicialmente recomendou um protocolo de irradiação com LED violeta de duração total de 30 minutos, e que o mesmo seja posicionado a uma distância de 8 mm da arcada dental (Kury et al., 2018). Especula-se que o LED violeta seria compatível com o pico de absorção de luz dos pigmentos extrínsecos normalmente aderidos à superfície do esmalte dental (Zanin et al., 2016, Rastelli et al., 2018); contudo, os estudos são escassos ao reportar a penetrabilidade da luz através do esmalte e não há investigação acerca da homogeneidade do feixe de luz e da estabilidade de irradiância do mesmo ao longo dos 30 minutos de irradiação, fatores estes que podem tornar-se imprescindíveis para eficácia destes protocolos.

Os primeiros estudos *in vitro* demostraram que o protocolo proposto apenas com LED violeta acarretou alterações de cor perceptíveis em dentes artificialmente pigmentados com chá preto, mas significativamente inferior aos resultados estéticos alcançados por géis de HP (Gallinari et al., 2019, Kury et al., 2020b). Um estudo mostrou que o LED violeta sozinho apresentou maior eficácia em superfícies de esmalte pigmentada artificialmente com vinho, café ou fumaça de cigarro, mas que este resultado clareador foi comprometido em dentes bovinos livres de pigmentos extrínsecos e submetidos à profilaxia (Kobayashi et al., 2021). Todavia, algumas pesquisas reportaram que o LED violeta foi capaz de exacerbar a eficácia clareadora de géis comerciais em menores concentrações de HP (Fernandes et al., 2021, Costa et al., 2022). Também, há

relato de que o LED violeta não aumente os efeitos citotóxicos causados pelo peróxido de hidrogênio difundido até à câmara pulpar (Ribeiro et al., 2022a).

Ensaios clínicos randomizados e controlados, com acompanhamento de até 1 mês após a terapia clareadora, corroboraram que o LED violeta sozinho exerceu efeito clareador mínimo (Brugnera et al., 2020, Kury et al., 2020c, Mayer-dos-Santos et al., 2022). Entretanto, dois ensaios clínicos mostraram que a fonte luminosa aumentou significativamente a alteração de cor provocada por 37% CP, alcançando significativamente a alteração de cor promovida por 35% HP, mesmo com apenas 1/3 da concentração de peróxido no gel clareador comercial utilizado. A combinação do LED violeta com 37% CP, ainda, gerou risco de sensibilidade dental estatisticamente inferior aos protocolos com 35% HP (Kury et al., 2020c, Santos et al., 2021). Vale ressaltar, porém, a necessidade de acompanhamentos longitudinais destes ensaios clínicos e de avaliação colorimétrica utilizando índices de alteração de cor e de clareamento atualizados, o que não foi utilizado em nenhum dos estudos citados acima.

Frente à possibilidade da utilização segura do LED violeta e do aumento da eficácia de géis clareadores menos concentrados, a incorporação de fotocatalisadores nestes agentes poderia favorecer a geração ou decomposição de HP em ROS (Tano et al., 2012) que interagiriam em maior intensidade com os cromóforos dentinários, assegurando a melhor eficácia de géis clareadores menos concentrados. Neste contexto, há relatos prévios da incorporação de nanopartículas de dióxido de titânio (TiO_2) dopadas com nitrogênio, comerciais e obtidas pelo método de calcinação, em géis de HP a 6 e 15% e irradiados com LED azul (Cuppini et al., 2019, Thacker et al., 2021). Embora um ensaio clínico randomizado tenha reportado a diminuição significativa da sensibilidade dental resultante do clareamento em consultório com menores concentrações de HP associadas ao TiO_2, os resultados estéticos não foram mantidos (Bortollato et al., 2016).

Sabe-se que o dióxido de titânio tem absorção óptica à luz ultravioleta, mas que a sua dopagem com outros elementos químicos pode alterar a faixa de absorção para luz visível (Miguel Pelaez et al., 2012). Porém, outros fatores como o tamanho da partícula, rota de síntese, fases do TiO_2 (anatase, rutilo e brokita) e tendência à aglomeração também podem influenciar sua eficácia como agente semi-condutor do HP (Wu et al., 2019), principalmente em um gel clareador formado por uma rede polimérica

hidrossolúvel. Recentemente, uma nanopartícula de TiO_2 co-dopada com nitrogênio e flúor obtida por meio de um processo solvotérmico, apresentou tamanho médio de 10 nm, predominância da sua fase mais estável (anatase) e o dobro da absorbância à irradiação violeta em comparação a uma nanopartícula de TiO_2 comercial (Esteban Florez et al., 2018, Esteban Florez et al., 2020). Tal nanopartícula foi incorporada em adesivos dentinários comerciais, apresentando não apenas efeito antibacteriano devido à capacidade de geração de ROS, mas efeitos bioativos a longo prazo (Esteban Florez et al., 2018, Hiers et al., 2022). Sendo assim, a incorporação destas nanopartículas em géis clareadores com concentração reduzida de HP e irradiados com LED violeta tornar-se-ia mais um fator auxiliar na manutenção dos excelentes níveis de eficácia do clareamento em consultório e das propriedades de superfície do esmalte e biológicas da polpa dental.

Em vista do exposto, o objetivo desta tese foi caracterizar e avaliar a penetrabilidade e eficácia *in vitro* do LED violeta frente a diferentes tipos de pigmentações extrínsecas (*Artigo 1*) e conduzir o acompanhamento longitudinal de um ensaio clínico randomizado e controlado de clareamento em consultório com LED violeta e géis comerciais de CP e HP utilizando índices colorimétricos atualizados (*Artigo 2*). Ainda, esta tese avaliou a incorporação de nanopartículas de TiO_2 co-dopadas com nitrogênio e flúor em géis experimentais com concentração reduzida de HP e combinados com LED violeta, e avaliou seus efeitos na eficácia clareadora, pH do gel e nas propriedades físicas e biológicas da estrutura dental (*Artigos 3, 4 e 5*).

2. ARTIGOS

2.1 Artigo 1. Characterization and Effectiveness of a Violet LED Light for In-office Whitening

Short Title: Violet LED Whitening: Characterization and Effectiveness

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ABSTRACT

Objectives: This study characterized a violet LED light (V-LED; Bright Max Whitening) tooth whitening device and evaluated its efficacy on stained enamel compared to hydrogen peroxide (HP).

Materials and Methods: Characterization of the V-LED beam profile was performed using a laser beam-profiler. The irradiance was measured throughout an exposure cycle at 0- and 8-mm distances using an integrating sphere and a spectral radiometer. Bovine enamel/dentin blocks stained with black tea (BT), cigarette smoke (CS), or without staining (CONT) were subjected to: V-LED or 40% HP (n=10/group). Color parameters (ΔL, Δa, Δb, and ΔE_{00}) were measured using a digital spectrophotometer. Light transmission was estimated through 1-mm thick bovine enamel slices (n=5). ΔL, Δb, ΔE_{00}, and irradiance were analyzed by two-way ANOVA's and Tukey's tests, Δa by Kruskal-Wallis and Mann-Whitney tests, and light transmission by t-test (α=5%).

Results: Heterogeneous beam distribution was observed for the emitting V-LED chips. After 20 sequential exposures, irradiance levels were reduced 25-50%, regardless of the distance from V-LED. Localized irradiance values were statistically different between beam locations and different distances from the target. V-LED produced lower ΔE_{00}, ΔL, Δa, Δb values than HP for CONT and BT, with no differences for CS. Light transmittance decreased approximately 98% through 1-mm thick enamel.

Conclusions: V-LED irradiance was heterogeneous and decreased throughout the exposure cycles and was also greatly reduced with increasing tip distance. V-LED produced a significantly lower whitening effect on BT and control teeth.

Clinical relevance: This study contributes to the knowledge of V-LED and its clinical use.

Keywords: tooth bleaching, dental enamel, violet LED light, irradiance, and staining.

INTRODUCTION

Light-assisted, chair-side tooth whitening is a technique often used by dentists, and several light devices are available designed to enhance whitening results [1]. However, recent evidence implies that neither halogen lights nor blue LEDs enhance the final outcome of whitening when used with hydrogen peroxide (HP) or carbamide peroxide (CP) [2]. In view of this fact, the maintenance of this combination is debatable as negative effects were described in the literature, such as higher intrapulpal HP concentration [3]. Also, the application of whitening gels on enamel is sufficient to cause trans-enamel/dentin penetration of HP into the pulp chamber [4], which has been demonstrated to affect pulpal tissue viability [5]. Moreover, a decrease in microhardness [6], bond strength [7], and an increase in surface roughness of enamel [8] promoted by whitening gels were previously reported *in vitro*. Thus, alternative techniques for decreasing these adverse effects should be investigated in order to improve the safety of vital tooth whitening.

The use of LED technology at a visible violet wavelength for the purpose of whitening teeth in the absence of gels [9] is currently in use. This new generation of LED device presents a curved acrylic tip with light-emitting diodes capable of illuminating all teeth at the same time [10]. Sparse literature is available regarding the effectiveness of this technique; however, recent studies show that violet LED (V-LED) exposure, by itself, promoted whitening or increased the effectiveness of peroxide gels [11-13]. According to some authors, the wavelength of the V-LED (405-410 nm) could correspond to the absorption peak of chromophore molecules in surface stains [12,14]. Because these molecules are highly reactive, it is speculated that the presence of violet light could trigger instability of the chemical bonds, leading to a breakdown of bonds and promoting the whitening effect by a physical process [15].

Among the reports on the ability of V-LEDs to promote color changes is an evaluation of lessening stains left by black tea [11, 16]. The authors observed that V-LED alone was capable of changing tooth color, but to a lesser extent than HP or CP [11, 16]. The whitening effect of V-LED exposure was hypothesized to be the breakdown of red and yellow polyphenolic chromophores of the tea [16]. Therefore, the ability of the V-LED to whiten may be associated with the type of extrinsic pigment residing on the external

dental structure, because it is well-known that violet light has low penetration through enamel [17-19]. Thus, understanding the effect of V-LED light for whitening of different stains adhered to the enamel outer surface would be a primary step in understanding the mechanism of action of this light. In this context, research demonstrates that cigarette smoking results in deposition of chemicals on enamel surfaces [20], which can absorb violet wavelength energy [21]. Thus, evaluating the effectiveness of violet light on cigarette smoke staining, along with the determination of the depth to which violet light can pass through enamel, might clarify the rationale of violet light enamel whitening effectiveness.

Also, the determination of both the irradiance and beam profile characteristics of the V-LED whitening light unit is paramount in determining its interaction with external discoloration. Such analyses have been described for conventional light units [22-24], providing essential information for the quality of resin composites light curing. However, currently, there is no data characterizing V-LED used for tooth whitening and measuring the continuity of irradiance levels throughout the exposure cycle from such a commercial device. In addition, there is no information on the homogeneity of the emitted beam across the device's emitting surface, which could indicate the quality of the light source and, consequently, its potential for predictable tooth whitening capability.

The purpose of this study was to characterize a commercially available V-LED light designed for vital tooth whitening (Bright Max Whitening, MMOptics, São Carlos, SP, Brazil) and to evaluate the effectiveness of the V-LED to minimize enamel staining produced by cigarette smoke (CS) and black tea (BT), compared to the use of a conventional, commercial hydrogen peroxide agent. Additionally, measurement of the violet light transmitted through enamel was performed. The research hypotheses tested were that (1) the mean irradiance of the V-LED light source would significantly decrease during a recommended exposure interval, or among repeated, sequential exposures; (2) increasing the tip-to-target distance up to 8-mm would result in significantly lower irradiance than the value observed at the emitting end of the V-LED light unit; (3) exposure to the V-LED would provide significantly less whitening effect compared to a conventional HP whitening gel, regardless of the type of enamel staining, and (4) violet light would be significantly attenuated in irradiance by passage through a 1-mm thick enamel section.

MATERIAL and METHODS

Overall experimental design

This study was divided into three phases. The first phase (*Light characterization*) quantified different aspects of the V-LED unit (Bright Max Whitening, MMOptics, São Carlos, SP, Brazil): stability of irradiance during a single exposure cycle and over repeated, sequential exposures, the effect of increasing tip-to-target distance, and the uniformity of irradiance within the V-LED beam profile. The V-LED light was characterized according to the spectral irradiance and total irradiance at the emission end and at 8-mm distance, the capability to provide a consistent irradiance during the exposure time, and the uniformity of emitted beam at the tip end.

The second phase (*Whiteness evaluation*) evaluated colorimetric changes on bovine enamel stained with black tea or cigarette smoke after whitening with the V-LED unit or when using a standard hydrogen peroxide gel, with no additional light treatment. This phase included a total of 60 enamel/dentin blocks of bovine teeth submitted to distinct *"staining"* protocols (n=20/protocol):

I. Black tea solution (BT)
II. Cigarette smoke (CS)
III. No staining control (CONT)

After staining, specimens were submitted to one of the following *"whitening"* protocols (n=10/protocol):

I. V-LED irradiation (V-LED)
II. High-concentrated hydrogen peroxide (HP)

Figure 1 illustrates a diagram of the research's second phase. Color parameters of each tooth prior to and after whitening procedures were obtained using a hand-held spectrophotometer. Color changes as a result of treatment (ΔE, ΔL, Δa, and Δb) were then calculated.

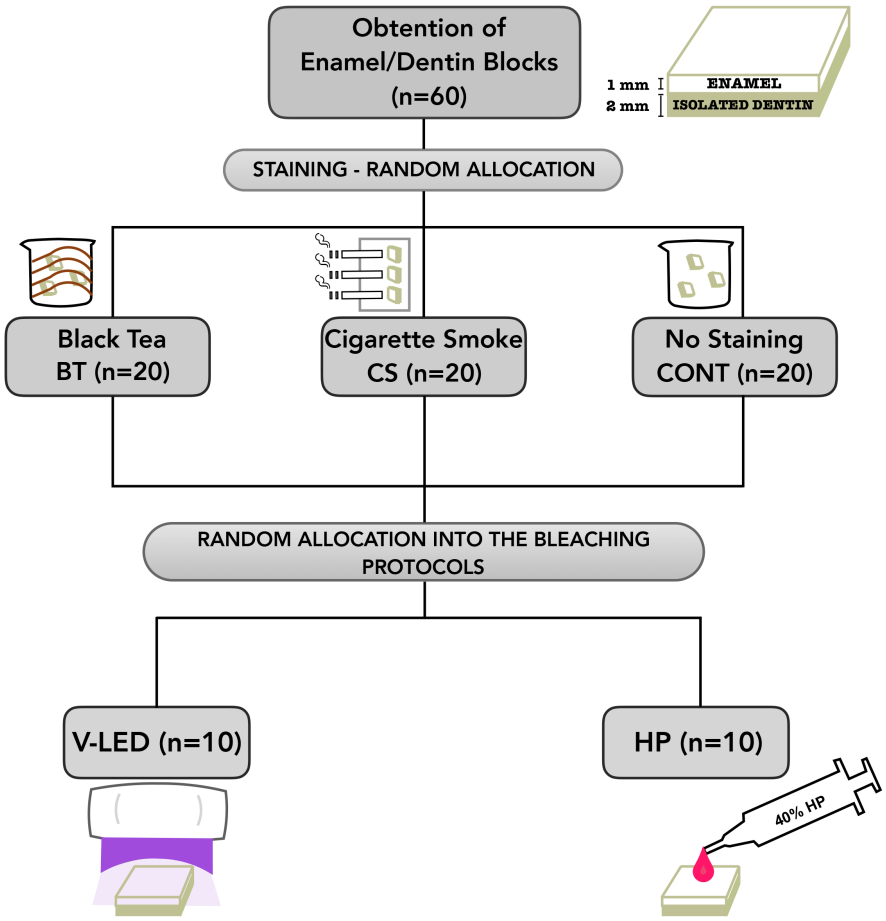

Fig. 1 Diagram illustrating the second phase of the research (*Whiteness evaluation)*

In the third experimental phase (*V-LED light transmission*), the light transmittance of the V-LED wavelength through controlled thicknesses of bovine enamel slices (n=5) was assessed.

Spectral irradiance and Beam profiling

The V-LED unit contains 4 LED sources with two emitting chips each. The spectral power of the emitted light was measured using a calibrated 6” integrating sphere (LabSphere, N. Sutton, NH, USA) and a spectral radiometer (USB 2000+, Ocean Optics, Dunedin, FL, USA). The power distribution profile of the emitted beam at the acrylic surface of the V-LED unit was evaluated using a laser beam-profiler (Model LBA-FW-SCOR20, Spiricon Inc., Logan, UT, USA). First, the beam profile was recorded without a target, and the light distribution across the emitting end was directly visualized. Then, a

second beam profile was recorded using a target (a square glass grounded with 1500 grit abraded glass plate [DG2X2-1500, ThorLabs, Newton, NJ, USA]) on one side. The resulting images of the beam profiles were collected using a software (LBA-FW SCOR Software, Spiricon, North Logan), where a rectangular area confined the beam analysis to capture the four emitting LED source emissions. The software used the power measured over this rectangle to calculate the pixel intensities and distributed power levels over the entire emission area. Different scales of irradiance were obtained for profiles with or without the target, in which white and “warm” colors represented high irradiance values in comparison to “cold” colors, representing lower irradiance levels.

Violet LED irradiance and change in output during an exposure cycle

The irradiance of the V-LED was determined using a spectral radiometer (USB 2000+, Ocean Optics, Dunedin, FL, USA), attached to an integrating sphere (CTSM-LSM-60-SF, Labsphere Inc., N. Sutton. NH, USA) and specific software (Spectra Suit STVS-VIS, Ocean Optics). The spectral irradiance accuracy of this system had been calibrated using methods traceable to NIST. The spectral irradiance profile, as well as the total irradiance, were measured at 0-mm distance from the radiometer located at the spot corresponding to the position of the right upper central incisor (RT). Measurement at this location (n=3) was used to determine the optimal irradiance of the device during the V-LED whitening protocol recommended by the manufacturer, consisting of twenty sequential 1-min exposures interrupted by 30 s intervals of no exposure, for a total of 30 minutes. Next, to simulate the clinical application (Figure 2 A to C), the tip of the V-LED was positioned at the manufacturer-recommended maximum distance of 8-mm from the target (integrating sphere aperture), and the same irradiation cycle was performed.

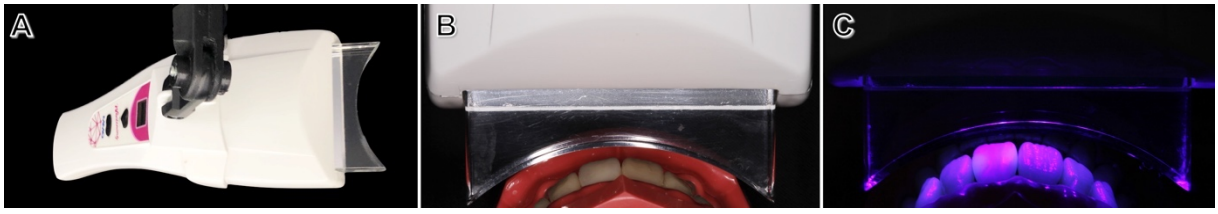

Fig. 2 Images of the V-LED light device: (A) The unit presents a curved acrylic tip coupled with the device’s body. (B) The position of the light-emitting tip end held against the maxillary anterior arch of a dentiform model, note the manufacturer-recommended maximum tooth-to-tip distance is 8-mm. (C) Same image as in panel “B”, but with the V-LED turned on, illuminating all anterior teeth at the same time

Additionally, irradiance was measured at two distinct locations, which simulated the location in the upper arch of RT and left central (LT) incisors at 0-mm and 8-mm distance from the radiometer. The 8-mm distance was applied because this is recommended by the V-LED's manufacturer when performing the whitening sessions in the patients. These measurements were repeated five times (n=5), but only at the beginning of the V-LED irradiation cycle. The RT and LT spots were determined based on a printed template with the average upper arch's teeth locations. The template was temporarily positioned on the surface of the acrylic tip. The diagram in the Figure 3 illustrates the use of the template. A mark was made in the middle of RT and LT locations. These exact locations were positioned towards the aperture of the spectral radiometer in order to detect the corresponding irradiance, at a 0- or 8-mm distance from the aperture.

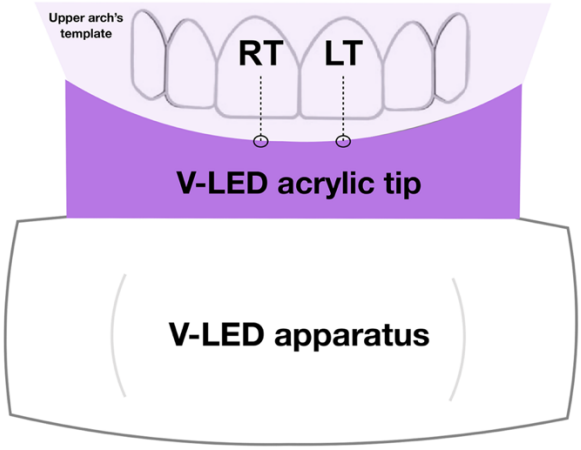

Fig. 3 Diagram representing the method used to determine the location of the acrylic tip corresponding to the point in which the V-LED would irradiate the right (RT) and left (LT) upper central incisors. The template was removed, and these marked locations were positioned towards the aperture of the spectral radiometer at a 0 or 8-mm distance. In this manner, the profile of power distributed to these two tooth locations was measured

Specimen preparation

Anterior bovine teeth were collected and stored in 0.1% thymol solution for no longer than one month. Teeth were cleaned using a periodontal scaler and were submitted to air abrasion using sodium bicarbonate to remove extrinsic staining. Sixty blocks of enamel/dentin (each enamel outer surface = 25 mm^2) were obtained from the buccal surfaces by sectioning the crowns with a water-lubricated diamond saw (Isomet-Buehler, Lake Bluff, IL, EUA). The blocks were fixed in an acrylic holder with the enamel surface

positioned downwards and the dentin surface, upwards. Then, the free surface of dentin of each block was abraded with a sandpaper (#600, Norton, Guarulhos, SP, Brazil) using a rotary polisher (Arotec, Cotia, SP, Brazil) under copious irrigation until the thickness of dentin reached 2 mm. The final thickness of the dental blocks was checked with a digital caliper (Mitutoyo Co., Kanagawa, Japan). The thickness of each block was equal to 3.0 mm (Enamel = 1.0 mm; Dentin = 2.0 mm). One-third of the blocks remained unstained (the experimental control) and were stored in freshly prepared artificial saliva [1.5 mM calcium ($CaCl_2$), 0.9 mM phosphate (NaH_2PO_4), 0.15 mM KCl, pH 7.0] [25, 26]. The solution was renewed every two days. The remaining teeth were submitted to black tea or cigarette smoke staining, as described below.

Staining protocols

The dentin surfaces of the dental blocks were isolated using nail polish to prevent contact with the stain. For BT staining, two grams of black tea (Leão, São Paulo, SP, Brazil) were placed into boiling water (100mL). Five minutes after infusion, the tea was cooled to room temperature (22^{o}C). Thus, only the enamel portions of the blocks (n = 20) were exposed to the tea, placed in the black tea solution for 24 h [27] and stirred every four hours. Following the 24 h-immersion, the stained blocks were rinsed in deionized water, sonicated in deionized water for 5 min, and stored in artificial saliva at 37^{o}C up to the beginning of whitening protocols.

An additional set of dental blocks (n = 20), used for CS staining, were fixed in utility wax in an acrylic holder leaving only the enamel surface exposed. This holder was placed in a machine that simulates smoking that was developed by the Operative Dentistry Division, Department of Restorative Dentistry, Piracicaba Dental School-UNICAMP (No. 01810012043 INPI, National Institute of Industrial Property). The machine simulates the smoking behavior usually performed by a smoker, with the smoke remaining in contact with specimens for 3 s. The mechanism consists of inhaling the atmospheric air every 10 s, and the smoke produced is kept in contact with the enamel surface of the specimen, followed by exhalation. The blocks were submitted to one pack of cigarettes (Marlboro, Philip Morris Brazil, Santa Cruz do Sul, RS, Brazil) a day for five consecutive days [28].

Between cycles of exposure, and upon completion of the total treatment, the specimens were stored in the artificial saliva, similarly to the tea-stained blocks.

Whitening protocols and colorimetric evaluation of tooth surfaces

The dental blocks were subdivided (n=10/treatment group) and submitted to either exposure to the V-LED or HP-whitening treatment. Twenty 1-minute exposures to the V-LED were performed, allowing a 30s non-exposed interval between exposures [26]. A total of 8 sessions were performed on each treated block. The buccal enamel surfaces of the blocks were firmly positioned upward and 8 mm distant from the V-LED acrylic tip. Specimens were rehydrated continuously by using moist gauze during the intervals. Figure 2 illustrates the shape of the V-LED acrylic tip as well as its ideal position towards teeth. The illuminated area comprises a wide reach of the light of both upper and lower arches, as it is possible to observe in the picture using a dentiform. The HP group was treated with a 40% HP gel (0.1g, Opalescence Boost, Ultradent, South Jordan, UT, USA) applied on the enamel surface for 15 min, rinsed thoroughly, followed by a second application. This procedure was repeated a second time after 72 h. The treated blocks were stored in artificial saliva at room temperature among the sessions.

The colorimetric evaluation of the enamel in each treatment group was performed before (T_0) and after (T_B) the whitening therapies using a digital hand-held spectrophotometer (EasyShade, Vita Zahnfabrik, Bad Säckingen, Germany) with the active point of the device in the center of each specimen. Four repeated measurements were made and the average of the color parameters were recorded (L*, a* and b*) and ΔL, Δa, Δb values were calculated. The color change of enamel (ΔE_{00}) was calculated using the CIEDE2000 system [29], as follows:

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + RT.\left(\frac{\Delta C'}{K_C S_C}\right).\left(\frac{\Delta H'}{K_H S_H}\right)}$$

Light transmission through enamel

One-mm thick enamel slices (n=5) were obtained from freshly extracted bovine teeth using a diamond saw (Isomet-Buehler, Lake Bluff, IL, EUA). The slices were wet-polished using SiC abrasive polishing paper (#600, #1200 grits) and positioned over

a flat, thin holder having a 3.3 mm diameter hole in the middle. The holder was positioned in the center of the integrating sphere previously mentioned, which was connected to the same light-measurement system previously described. A high-power, plasma arc LCU (sn6000, Arc Light II, Air Techniques Inc., Hicksville, NY, USA) was used in this analysis. The tip of the plasma arc light was placed in contact with the 1-mm thick enamel specimen.

Statistical Analyses

The data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests ($p>0.05$), respectively. The ΔL values were transformed into Log10 and ΔE_{00}, ΔL, Δb and irradiance (RT and LT central incisor locations) values were evaluated by two-way ANOVA and Tukey *post-hoc* tests. The Δa values were compared using Kruskal-Wallis and Mann-Whitney tests. Light transmission through enamel was analyzed by a Student t-test. The statistical analyses were performed using software (Version 15.0, SPASS, IBM SPSS Inc., Armonk, NY, USA) using a pre-set alpha of 0.05.

RESULTS

Spectral irradiance

The spectral irradiance of the V-LED device is shown in Figure 4. Irradiance was detected within the violet wavelength range. The maximum peak observed was at 401.82 nm. The total power of the V-LED was 1.202 mW, and the total radiant emittance 137.4 mW/cm^2.

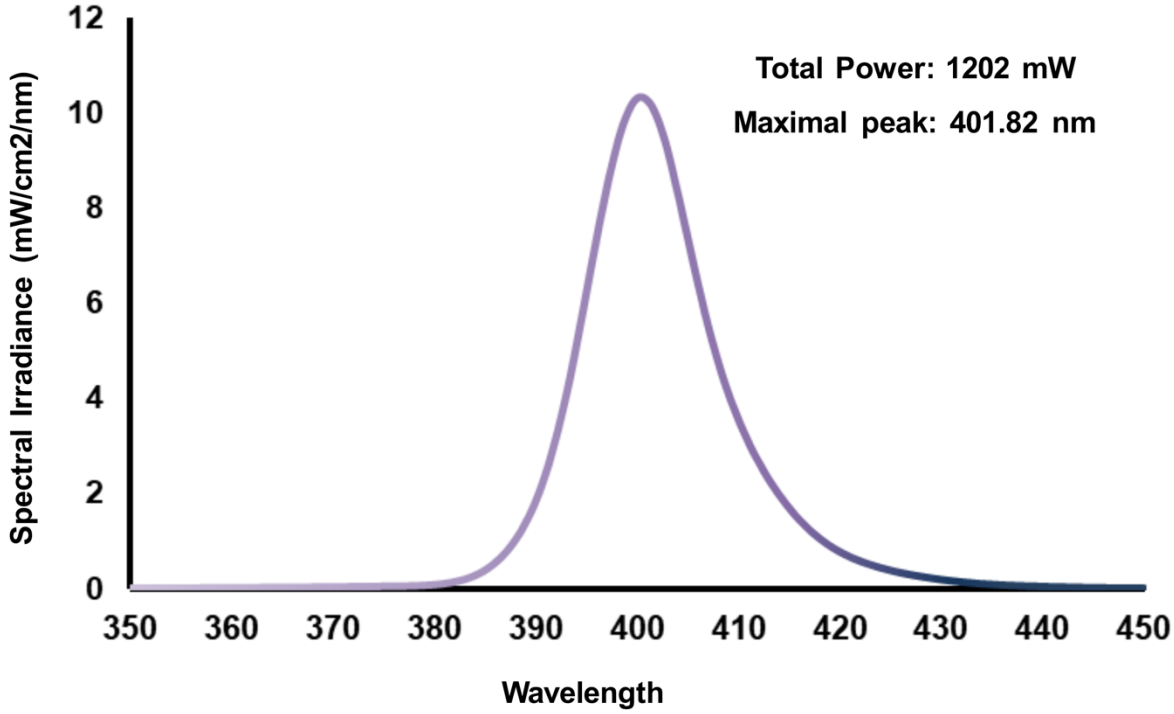

Fig. 4 The spectral distribution of violet light emitted by the V-LED device at a 0-mm distance

Beam profiling

The power contained within the rectangular beam area was 1.2 Watts (1,200 mW). The non-targeted beam profile of the V-LED unit exhibited a very heterogeneous distribution of irradiance from the V-LED chips across the emitting end of the curved acrylic tip. Figure 5 illustrates the distribution of the chips along the acrylic tip, turned off (Figure 5 A) and turned on (Figure 5 B). The location of the chips is observed in the center of each light unit body. Violet light is emitted through the chips, and reflection of this light is seen in the entire peripheral areas (Figure 5 B). The heterogeneity in light output of each chip, especially when the target was not used, is illustrated in Figure 5 C - D. Analysis

of the 2-D beam profile revealed that the V-LED chips produce localized spots with high energy represented by red and white colors, presenting irradiance values near 910 mW/cm^2 (without target, Figure 5 C) and 410 mW/cm^2 (with a target, Figure 5 E).

It is noteworthy that only the emitting areas of the V-LED chips (peripheral and central regions) exhibit irradiance at least equal to the values noted in the blue areas (~200mW/cm^2) or higher (Figure 5 C). The white areas indicate pixel saturation (values greater than ~1000mW/cm^2). Moreover, analysis of the 3-D beam profile shows “flat” areas receiving low irradiance, ranging from 110 to 910 mW/cm^2 (without target, Figure 5 D) and from 110 to 510 mW/cm^2 (with a target, Figure 5 F). Imaging of the light falling on a target indicated a better distribution of irradiance, because the whole area of the chips presented irradiance compatible with at least the intensity levels of the blue areas (Figure 5 E). However, the presence of a target (Figure 5 E - F) resulted in a lower irradiance, and the presence of higher and “warmer” (higher intensity) locations in certain areas were maintained.

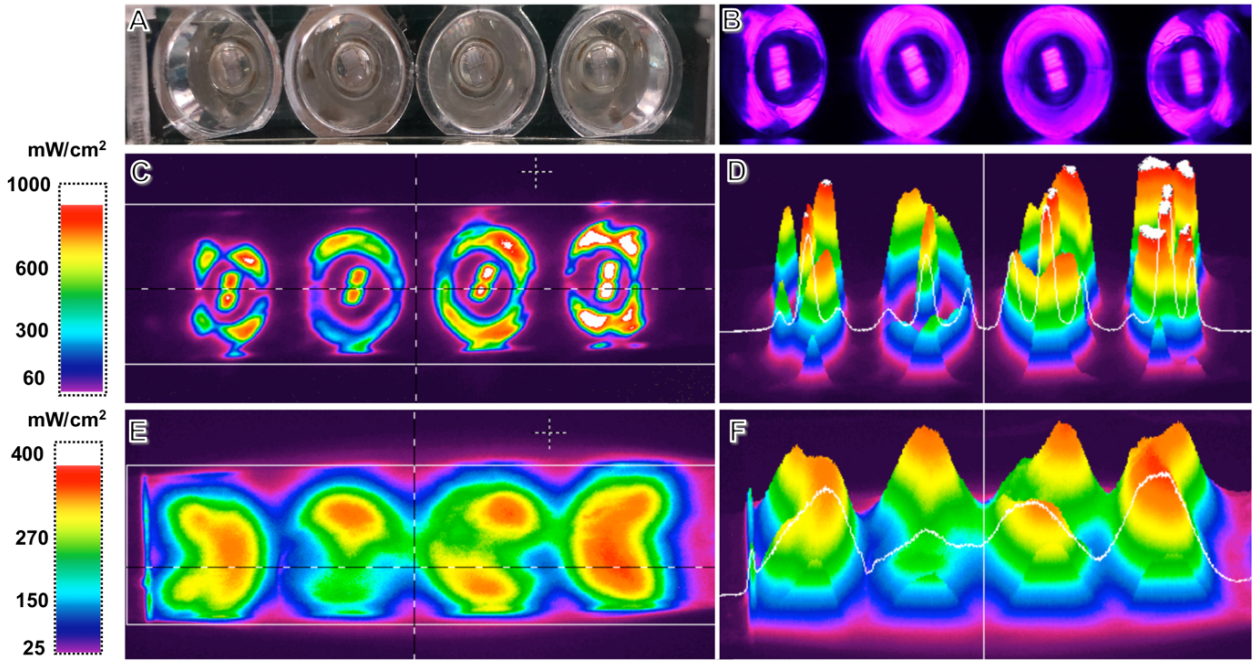

Fig. 5 Images of the V-LED device tip (A chips off and B chips on) and beam irradiance profiles of the light-emitting diodes of the V-LED light source without (C [2-D] and D [3-D]) or with (E [2-D] and F [3-D]) the ground glass target in place.

Irradiance with sequential exposures, simulating clinical use

The mean irradiance values during a complete whitening cycle (at the beginning and at the end of the first and last one-minute exposures) with the corresponding percentage of irradiance are displayed in Figure 6. Regardless of the distance of the device, the irradiance dropped after the first minute of light exposure. The subsequent nineteen exposures exhibited the same behavior: the irradiance was not able to reach the initial value and continuously dropped at the end of the minute-long exposure in a spear-shaped format, even after 30 s of cooling between sequential exposures. During the last exposure, the 0-mm tip to target distance exhibited 75% (beginning) and nearly 50% (end) of initial irradiance. At the 8-mm tip-to-target distance, there was a 25% reduction in irradiance relative to that of the beginning of the first exposure and approximately a 50% reduction after the twentieth irradiation, compared to the first irradiation. Also, at all the exposures, the irradiance at 8-mm tip-to-target distance was lower than at 0-mm distance.

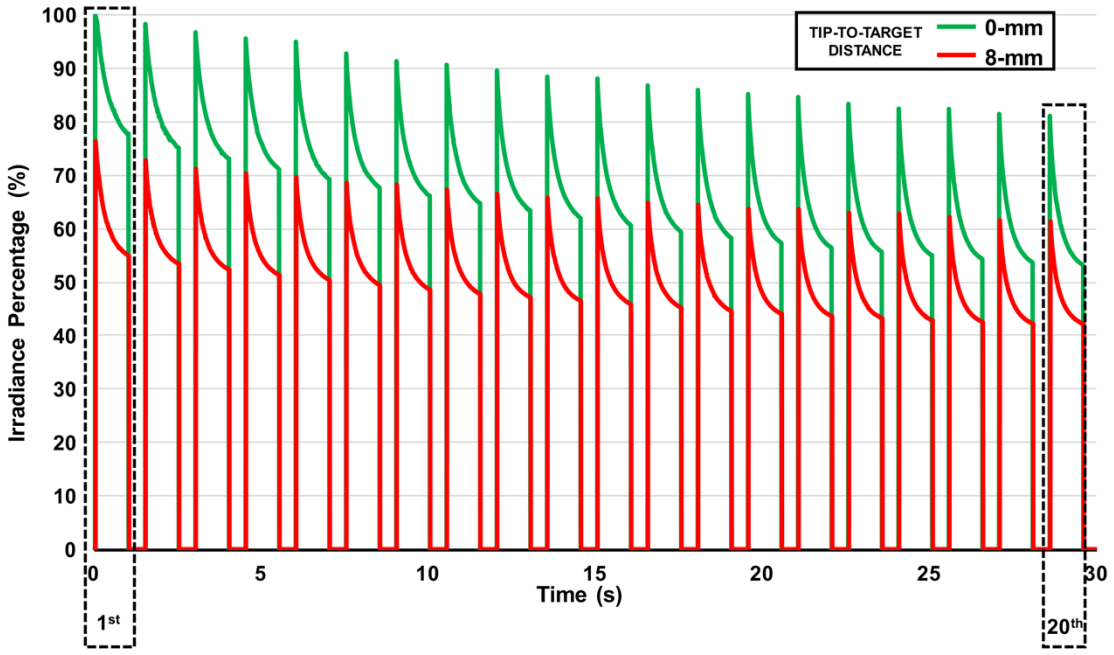

Fig. 6 Irradiance of V-LED light (according to the values described in Table 1) at 0 mm (green line) and 8 mm (red line) tip-to-target distances after a total of twenty 1-min exposures, each followed by a 30 s non-emission interval. The irradiance values are presented in mW/cm^2 with their corresponding percentages. The highest irradiance value (10.8) was recorded at the 1st exposure at 0-mm distance (green line), representing 100% of the irradiance detected. The squares indicate the first and last exposures.

Table 1 depicts the values of irradiance (mW/cm^2) corresponding to RT and LT. The results indicated that location (RT or LT) of the central incisors and the tip-to-target distance statistically affected the irradiance of the V-LED ($p=0.003$). The RT central incisor received significantly higher irradiance than the LT, regardless of the distance to the light source ($p<0.05$). The 8-mm tip to target distance promoted significantly lower irradiance for both locations (RT and LT).

Table 1. Mean and standard deviations of irradiance (mW/cm^2) at different locations representing RT and LT upper central incisors, at two distances (0 and 8 mm).

Location	Irradiance (mW/cm^2)	
	0-mm	8-mm
RT	164.9 (6.1) ^{Aa}	116.3 (2.6) ^{Ab}
LT	124.3 (1.6) ^{Ba}	92.7 (15.0) ^{Bb}

Means followed by similar letters (upper case: location; lower case: within distance) are not significantly different.

Tooth Color change

Table 2 displays mean/median values of color parameters (ΔL, Δa and Δb) and color change (ΔE_{00}). According to the two-way ANOVA, “staining” and “whitening protocols” statistically influenced the results ($p<0.001$) for the color parameters of ΔE_{00}, ΔL, and Δb while the Kruskal-Wallis test detected significant differences for Δa among groups ($p<0.001$).

HP whitening presented higher luminosity differences (ΔL) and color changes (ΔE_{00}) than whitening with V-LED on enamel unstained (CONT) or stained with BT ($p>0.05$), but no significant differences were observed for CS–stained teeth treated with either V-LED or HP ($p=0.508$). The HP groups presented lower color parameters (Δa, Δb) for CONT, and only this group produced higher Δb values than V-LED on CS-stained teeth ($p<0.05$).

Table 2. Mean and standard deviation (ΔL, Δa and Δb) or median values and maximum; minimum values (Δa) of color parameters for enamel stained with black tea (BT), cigarette smoke (CS), or no staining (control - CONT) when submitted to whitening with V-LED and HP gel.

ΔL			
Whitening Protocol	CONT	BT	CS
V-LED	1.3 (4.5) ^{Bc}	10.3 (5.4) ^{Bb}	26.2 (9.8) ^{Aa}
HP	7.6 (2.0) ^{Ab}	26.9 (6.8) ^{Aa}	29.6 (11.7) ^{Aa}
Δa			
Whitening Protocol	CONT	BT	CS
V-LED	0.0 (-0.1;1.1) ^{Bc}	-3.2 (-5.2; -1.4) ^{Bb}	-6.7 (-7.8; -1.3) ^{Aa}
HP	-1.6 (-2,3; -0.1) ^{Ab}	-9.2 (-15.0; -1.9) ^{Aa}	-7.4 (-11.1; -3.7) ^{Aa}
Δb			
Whitening Protocol	CONT	BT	CS
V-LED	-2.3 (1.3) ^{Bb}	-3.6 (4.3) ^{Bab}	-7.1 (3.8) ^{Ba}
HP	-6.7 (2.1) ^{Ac}	-15.7 (7.3) ^{Aa}	-11.4 (3.0) ^{Ab}
ΔE_{00}			
Whitening Protocol	CONT	BT	CS
V-LED	2.6 (1.9) ^{Bc}	9.8 (3.7) ^{Bb}	21.0 (7.7) ^{Aa}
HP	6.0 (1.4) ^{Ab}	23.0 (6.4) ^{Aa}	23.9 (10.2) ^{Aa}

Means or medians followed by similar letters (Uppercase: whitening protocols (vertical); lowercase: staining mode (horizontal) are not significantly different, according to two-way ANOVA and Tukey Test or Kruskal-Wallis and Mann-Whitney. Delta (Δ) refers to $T_B - T_0$; ΔL refers to luminosity differences of the L* axis (0: black and 100: white); Δa and Δb refer to differences in the color parameters a* (+a*: reed; -a*: green) or b* (+b*: yellow and -b*: blue).

Overall, the whitening protocols produced higher color and luminosity changes (ΔE_{00}, ΔL), and lowered the color parameters (Δa, Δb) of the stained enamel (BT and CS) compared to the unstained group (CONT). The results also showed that V-LED whitening protocol produced higher color and luminosity changes (ΔE_{00}, ΔL, Δa) on CS-stained teeth compared with the BT-stained or unstained groups ($p<0.05$).

Light transmission through enamel

Figure 7 shows that 1-mm thick enamel attenuated approximately 98% of the emitted light at a wavelength similar to that of the V-LED light (401 nm). The open aperture of the integrating sphere detected 100.0% of light transmission while the interposition of enamel detected only 2.3 ± 0.4 % of the light. There was a significant reduction (~ 98%) in irradiance when the 1-mm enamel slice was interposed ($p = 0.009$). Figure 7 displays

an example of irradiance detected at the specific wavelength of the V-LED light source (401 nm) on the violet line.

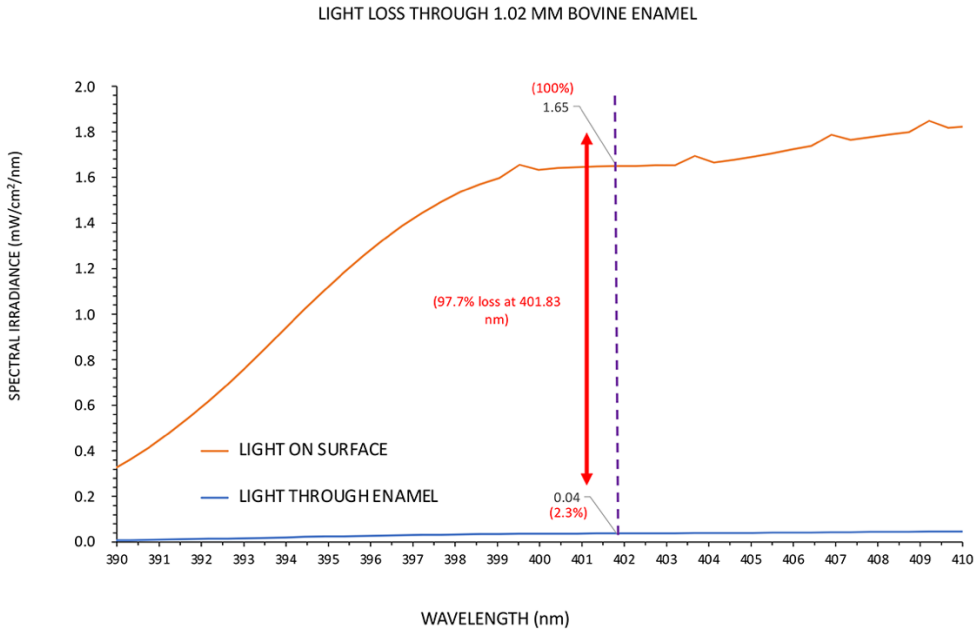

Fig. 7 The spectral profile of the broad-banded plasma arc light. At the emission wavelength of the V-LED whitening unit (401 nm), the maximum spectral irradiance of the PAC light is seen to be 1.65 mW/cm^2/nm at the enamel surface, and it is only 0.04 mW/cm^2/nm after passing through the enamel specimen

DISCUSSION

The first research hypothesis was accepted, as the irradiance of the V-LED unit significantly decreased during repeated, sequential exposures. Other than distribution, irradiance was also investigated throughout the V-LED activation cycle. The spear-shaped light emission profile seen in each 1-min long light activation (Figure 6) indicated that the V-LED source was unable to recover or maintain its irradiance during light activation in comparison to the 1st-minute values. Because LEDs generate significant thermal energy, heat dissipation technologies such as internal fans and metal heat sinks are necessary to remove heat and avoid damage to the light source [17]. Higher chip temperatures can affect the reliability of the unit by reducing the output from the LED [22]. Despite the continuous irradiance decrease during the 1 min irradiation, the 30 s interval recommended by the manufacturer for recovery did not provide enough time for the irradiance to return to its pre-rest values, probably due to incomplete chip cooling. The literature indicates that irradiance of many light-curing units is not affected in such a manner; however, the cooling down of devices is suggested among multiple uses [23], which is a limitation of the whitening V-LED because it lengthens the total exposure cycle time.

The evaluation of the V-LED device irradiance positioned at two distances away from the buccal surface of the teeth (0 and 8 mm) was determined because the manufacturer indicates that light should be 8-mm distant from the teeth. The results showed that increasing the tip-to-target distance to 8 mm resulted in a significant irradiance decrease (Table 1, Figure 6), and as a consequence, the second hypothesis was accepted. The effects of decreasing the irradiance as the tip-to-target distance increases have been documented for dental light-curing units [17, 23]. Despite the facts that cycling cooling intervals and distance of light decreased irradiance of V-LED, these measures could attenuate pulp temperature changes. Although pulp temperature of teeth submitted to V-LED whitening has not been described, it has been demonstrated that halogen, plasma arc, and LED lights could produce *in vitro* temperature raise ranging from 5 to 8^0C, surpassing the limit for potential pulp necrosis [17]. However, because of the low penetration of violet light through enamel, this aspect is not considered a significant causal factor.

The findings of tooth color change (Table 2) indicated that the V-LED was able to generate perceivable color change. However, the use of the V-LED generated the same whitening effectiveness as HP whitening therapy only when applied to cigarette smoke-stained enamel. Therefore, the third research hypothesis, that the violet exposure would not significantly reach the whitening effectiveness of HP whitening gel regardless of staining type, was accepted. Previous studies verified that the V-LED light alone was able to change the color of enamel stained with black tea, but to a lesser extent than 35% HP [11, 16]. However, the present study indicated that V-LED whitening is as effective as a high-concentration of HP under cigarette-staining conditions. The more significant increase in lightness (ΔL) and decrease in redness (Δa) for cigarette smoking may indicate that the violet irradiation removed or interacted more intensely with this type of stain than with the chromophores in black tea, a solution commonly used to standardize colorimetric evaluations [26]. Thus, the present findings suggest that one type of staining may be more susceptible to violet irradiation than others. Indeed, it has been speculated that the V-LED would match the absorbance peak of staining molecules, thereby removing them from enamel by a physical process [14,15]. Another possibility could be the deposition of smoking residuum, such as cadmium, on the enamel surface [20]. Some cadmium compounds absorb photons with energy corresponding to the violet and blue portion of the visible spectrum [21].

Light transmission through enamel revealed that 98% of violet light irradiance at the wavelength of the commercial V-LED tested was absorbed (Figure 7). Thus, the fourth research hypothesis was accepted. For light transmission through the enamel, the plasma arc was used because it emits a broad wavelength range over the visible spectrum, including violet light [18]. Use of this type of light was necessary because of the very low levels of light passing through the 1-mm thick specimens: too low for the 6” sphere to resolve [17]. However, previous to testing, spectral irradiance peak of V-LED light source was defined at 401.82 nm, and the results showed that 1-mm thick enamel is capable of absorbing violet light irradiation in this wavelength. Although little information exists concerning violet light transmission through the enamel, literature results suggest that the irradiance of a light-curing unit emitting blue wavelengths continuously decreased as enamel thickness increased (1 to 4 mm) [19]. Even though the present study did not

compare the influence of enamel thickness on the transmission of violet light, the collected data is sufficient to suggest that a thin layer of enamel is capable of absorbing (or scattering) significant levels of violet irradiation. Based on the importance of enamel thickness to the violet light whitening, the enamel was standardized at 1-mm thick for the whiteness evaluation. The control of dentin thickness was also an important factor due to the action of HP in dentin, wherein the HP by-products would break the intrinsic pigments [4].

The beam profile images of the emitted beam from the V-LED device demonstrate high levels of non-uniformity. Even though the radiant power indicated by the manufacturer (1.2W) [16, 26] was confirmed, the beam-profile images, both in 2-D and 3-D profiles, demonstrated the non-homogeneous distribution of irradiance across the tip of the light. Although the irradiance considers the area of the surface receiving the light, it only provides information of an average output [17], and it does not indicate the presence of areas with higher or lower irradiance, known as locations with “warm” or “cold” colors, respectively [18]. The measurement of the irradiance set at the RT and LT central incisors confirmed an irregular distribution of irradiance. To date, there are no reports in the literature of beam profile analysis in light sources used for in-office tooth whitening. The clinical implications of these local irradiance values can be of importance. If the intensity of light is a causative factor in the effectiveness of violet light to whiten a stain, then one might expect a non-uniform stain removal, if the light itself demonstrated a highly nonuniform spatial output.

The present study findings suggest that color change resulting from exposure to the V-LED alone may be clinically perceptible, because ΔE_{00} was higher than 0.8 in all groups: the minimal color change value that is visually detectable [29]. These data corroborate findings from other authors that also observed perceivable color change due to exposure to a V-LED alone [11, 16]. More pronounced whitening for stained enamel could corroborate the assumption that violet light matches with absorbance peak of various pigment types [13, 15, 30]. Thus, a clinical discoloration caused by cigarette smoke could benefit from application of this protocol. It is important to notice that the simulated protocol of cigarette smoke staining was based on a standard frequency of smokers, previously determined in another study [28]. Because previous research

demonstrated that cigarette smoking deposits chemicals (nickel, cadmium, and lead) on enamel surfaces [20], which could absorb violet wavelength energy [21], the behavior of violet LED whitening on this type of substrate is crucial for understanding the role of this novel technology in smokers. Also, because a randomized clinical trial pointed out that whitening with CP was stable in smoker patients after 12 months only following dental prophylaxis [31], alternative treatments for this type of staining could contribute to the whitening effectiveness in smokers. On the other hand, black tea staining was elected to evaluate the effect of violet light in at least two types of pigments, with the tea, in particular, simulating a dietary habit that enables the deposition of polyphenolic molecules instead [7]. Black tea staining for 24 h is sufficient to attain high levels of darkening before whitening procedures, being a commonly used protocol in studies evaluating the effectiveness of tooth whitening products [11,16].

Also, results showed that the violet wavelength demonstrates low capacity to diffuse through the enamel. Indeed, violet light was unable to pass through 1-mm thick enamel samples. Therefore, data indicates that the V-LED effect is probably only superficial, and light would possibly interact or cleave only extrinsic, surface pigments. In contrast, due to the known trans-dentinal hydrogen peroxide diffusion ability [4], it is expected that free radicals released by hydrogen peroxide will be more dynamic in removing pigments in deeper enamel and dentin structures. The data from the present study corroborate the findings of a randomized clinical trial, where non-smoking volunteers submitted to whitening only with a V-LED light presented a lesser whitening outcome than those treated with high-concentrated whitening gels [13]. The fact that the V-LED treatment (up to 10 sessions) is longer, along with the lower whitening effect compared to HP (up to 2 sessions), also represent a major drawback of V-LED technique.

Characteristics of the V-LED device also provided important information that could be extrapolated into clinical conditions. Firstly, the irregular distribution of the beam profile raises the concern that light irradiance would heterogeneously reach the surface of the teeth, thereby promoting irregular interaction of the light with the staining along the teeth. In this sense, the evaluation of beam profile using a ground glass target, which could work as a “homogenizer”, provided important information suggesting that irradiance

could be more regularly distributed if a homogenizer tip was used, which is in accordance with recent concepts developed for dental light-curing units [24]. Secondly, the length of the exposures and intervals cycle seems to affect the stabilization of the V-LED emission levels. However, the decrease of exposures and increase of interval time could affect the whitening outcomes as well as extend the number of sessions required for treatment, which is already a drawback of the V-LED protocol in comparison to the HP whitening protocol [16]. Finally, even though the manufacturer-recommended 8-mm distance to not overheat the pulp, the tip-to-target distance diminished the irradiance of the V-LED light. Perhaps this distance was also suggested to not significantly affect gingival tissues, which would also be in the V-LED path during exposures.

A limitation of this study was the color change evaluation, as staining in an *in vitro* condition is exacerbated in comparison to a clinical situation. Also, a staining depth evaluation along with light transmission could have determined the extent of the V-LED action on enamel color change. Pulp temperature evaluation could have addressed the necessity, or not, of increasing the device's distance from the teeth, as clinical trials have already demonstrated that tooth sensitivity is observed with V-LED whitening protocols [9,13]. Therefore, further investigations about the effect of pulp temperature variation should be conducted to provide information on the safety of V-LED use on patients. Further research could also improve the light emission stability and uniformity of the devices to ensure the quality of the whitening protocol.

CONCLUSIONS

Within the limitations imposed by the methodology used in this study, the following conclusions can be drawn:

1. The irradiance of the V-LED was negatively affected by the repeated sequential exposures and intervals and the 8-mm tip distance,
2. Even though V-LED exposure alone was able to produce a perceptible color change, it presented similar results to HP only when enamel was stained with cigarette smoke, and
3. About 98% of the V-LED light was not transmitted through 1-mm enamel.

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2.2 Artigo 2. Colorimetric evaluation after in-office tooth bleaching with violet LED: 6- and 12-month follow-ups of a randomized clinical trial

Short Title: Long-term follow-up after violet LED bleaching

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ABSTRACT

Objectives: To evaluate the long-term outcomes of in-office bleaching with violet LED light (LED) alone or combined with carbamide (CP) or hydrogen (HP) peroxides.

Methods: Volunteers of a previous short-term study were recalled for 6- and 12-month follow-ups, according to the following interventions (n=18/group): LED, CP, LED/CP, HP and LED/HP. The objective color (ΔE_{ab}, ΔE_{00}) and whiteness index (ΔWI_D) changes were calculated applying the CIELab coordinates' values obtained using a spectrophotometer. A visual shade guide determined the tooth's subjective color change (ΔSGU). Data were submitted to one-way ANOVA or Welch's ANOVA, following appropriate post-hoc tests (α=5%).

Results: The LED and CP groups exhibited the lowest ΔE_{ab}, ΔE_{00} and ΔSGU ($p<0.05$), but the LED group displayed a significantly lower ΔWI_D. After 12 months, the LED/CP group presented a higher ΔE_{ab} and ΔE_{00} than the CP group ($p<0.05$). ΔE_{ab}, ΔE_{00}, ΔSGU or ΔWI_D means did not differ statistically between the LED/CP and HP groups. The LED/HP group presented a higher ΔE_{00} than the HP group, regardless of the time.

Conclusions: The bleaching efficacy of LED alone was significantly lower compared to the LED/CP and HP-containing protocols. After 12 months, the LED/CP and HP groups did not differ in bleaching efficacy. LED irradiation only increased the objective color change of bleaching gels.

Clinical Relevance: LED alone promoted a long-term perceptible bleaching, but not compatible with that of high-concentrated HP. The bleaching outcomes of violet irradiation to 37% CP were maintained over time, with LED/CP demonstrating comparable results to HP even after 12 months.

Keywords: Tooth Bleaching, Hydrogen Peroxide, Carbamide Peroxide, Light, Follow-up.

INTRODUCTION

In-office dental bleaching is one of the most frequent esthetic treatments performed by dentists and is often indicated for fast chromatic and perceptible changes in the anterior teeth [1-4]. The bleaching gels available for in-office bleaching are composed of highly concentrated hydrogen (HP) or carbamide (CP) peroxides [5]. The main difference between these two gels is the composition of CP, which presents carbamide peroxide with urea. When these compounds come into contact with water, they break down into hydrogen peroxide and ammonium and, later, in oxygen-free radicals and water. Therefore, the CP gel contains only one-third of the total hydrogen peroxide concentration compared to the HP gel [6]. As a consequence, HP may display a higher bleaching efficacy than CP, taking into consideration the same application time [7].

In recent decades, several types of light sources have been used to attempt to increase the efficacy of in-office bleaching gels [8]. The rationale behind this approach is to heat the peroxide gels through the thermal energy generated from the light. As a consequence, the increase in the release of free radicals could enhance the bleaching effects [8,9]. Researchers have shown controversial bleaching outcomes in regard to the use of the argon laser, halogen lamp, blue LED or diode laser [10,11,12]. Such divergent outcomes could be explained based on the differences between irradiation protocols, time of use or irradiance and power stability of the different light sources. Also, differences in the bleaching gel's type, concentration or application regimen hinder the comparison among studies [10,15]. However, systematic reviews concluded that no light irradiation source was able to enhance the efficacy of in-office bleaching gels [8,13-15].

Some clinical reports have suggested the use of a new violet LED light for in-office dental bleaching [16-20]. In these clinical case reports, the violet LED was used in combination with CP and HP. However, the violet LED light manufacturer also recommends the irradiation of violet light without any peroxide or chemical agent [21]. Based on the lower light penetrability of the violet wavelength [22], some authors have speculated that the violet light alone possibly degrades the extrinsic staining molecules adhered to the enamel surface by a photocatalyst process [18,20].

It is worth mentioning that the violet LED bleaching protocols, combined or not with peroxide agents, have been applied without sufficient evidence to support their

efficacy and safety. Only three randomized clinical trials assessed the efficacy of violet LED and its effect on tooth sensitivity, either alone or combined with at least one type of peroxide agent (CP or HP) [23-25]. The effect of violet LED alone on tooth sensitivity was minimum. Also, the combination of violet light with 37% CP gel presented the same efficacy as that of the non-irradiated 35% HP gel, but with reduced tooth sensitivity [25].

Even though some clinical trials attest to the limited ability of violet LED to bleach without peroxides or to increase the efficacy of peroxide agents, these reports are part of short-term evaluations only (up to 14-day follow-up) [23-25]. Besides, there is no evidence for the clinical efficacy of violet LED irradiation protocols using updated colorimetric systems. None of the clinical trials reported the color change based on the CIEDE2000, a system that corrects discrepancies in the CIELab color change formula [26]. Moreover, the clinical application of the whiteness index for dentistry, an index developed specially to detect the whiteness levels of teeth and is more suitable to visual perception, ought to contribute to the understanding of the topic [27].

Therefore, this study determined the long-term colorimetric evaluation (6 and 12 months from bleaching procedures) from a randomized and controlled clinical trial of violet LED in-office bleaching protocols. The null hypotheses tested were that, after 6 and 12 months from bleaching, (1) violet LED light alone would not present the same bleaching efficacy as CP or HP gels, (2) violet irradiation would not increase the color and/or whiteness changes promoted by CP and HP and (3) irradiation of CP with violet LED would not result in a bleaching efficacy similar to that observed for high-concentrated hydrogen peroxide.

MATERIAL AND METHODS

Ethical Aspects

The ethical aspects of this clinical trial were approved under the registration numbers: 72879717.7.0000.5418 (Issuing authority: *Plataforma Brasil*) and 2.229.061 (Issuing authority: Local Ethical Committee). An amendment was also approved by the Local Ethical Committee (3.776.209). This research was registered in the National Clinical Trials Registry (REBEC - RBR-5t6bd9) and followed the CONSORT guidelines. This is a long-term evaluation of a previously short-term published study [25]. Patients included in this study signed informed consent in accordance with the Declaration of Helsinki before the first clinical session.

Recruitment of Volunteers

The patients were recruited from the University through announcement signs distributed into a few facilities of the building. Patients of dental clinics, dental students, faculties and staff were able to enroll for the initial appointment, which checked the eligibility criteria. Before signing the consent form, potential patients were clinically evaluated and were selected based on the inclusion or exclusion criteria found in Table 1.

Table 1. According to the criteria determined, the volunteers were either included or excluded from the clinical trial if they presented one of the conditions below

Inclusion	Exclusion
Age: 18 to 60 years old;	Enamel cracks;
Absence of carious lesions;	Previous dentin hypersensitivity;
Healthy gingival conditions;	Pregnancy;
Vital teeth;	Smokers;
Color of the canine's cervical/middle third should be at least A2;	Endodontically treated teeth and/or with extensive restorations (minimal restorations accepted);
It was mandatory to report availability to attend follow-up appointments.	Previous allergy to one of the materials planned to be used in the dental procedure;
Absence of edentulous space between maxillary and mandibular premolars.	Volunteers who have undergone bleaching during the last 3 years.

The number of patients was determined by a sample size calculation, using color change values from a published study [28]. Adopting a 5% level of significance, an 80% power and a 0.50 effect size *(f)*, the calculation indicated a minimum of 16 patients to detect differences among groups (BioStat, AnalystSoft, Walnut, CA, USA). Twenty volunteers were recruited, taking into consideration a further possibility of drop-out. The short-term study conducted by Kury et al., 2020, showed that 18 patients from each intervention group concluded the bleaching treatments and returned for the 14-day follow-up [25].

Randomization, Blinding and Allocation

A research member, not responsible for either treating or evaluating the patients, performed the randomization and allocation concealment of the volunteers (V.C.). A code written in an opaque and sealed envelope was assigned to each participant. Then, the envelopes were randomly distributed into the five intervention groups [24,28,29]. The randomization was open only to the operator, before the beginning of the bleaching intervention. Two members were directly involved with the clinical appointments: one operator (E.E.W) and one evaluator (M.K.). The operator was informed of which group each participant was allocated to because of the bleaching agents and light characteristics. However, the evaluator of the colorimetric analysis was blinded to the procedures. The volunteers were not aware of either the type or the concentration of the bleaching gel they were exposed to. For this purpose, any label, brand logo or packaging that would enable identification of the products was removed [30]. The evaluator was previously calibrated during the in-office bleaching appointments of five participants excluded from the clinical trial. This calibration was performed by measuring the color of the cervical and middle third from the buccal surface of the upper canines after each training bleaching session [29]. The operator was responsible for recording the data. Another research member, also blinded, was in charge of scheduling the follow-up appointments (S.S.P.).

Interventions (Bleaching Procedures)

The bleaching protocols were defined according to the bleaching gel (HP, CP or none) and light irradiation method (violet LED or none) used, and patients were randomly allocated into five different groups (n=18/group): LED, CP, LED/CP, HP and LED/HP. Table 2 displays the composition of the bleaching gels and the technical specification of violet LED. The bleaching protocols were as follows:

(LED): The complete LED irradiation cycle totals 30 minutes (twenty 1-min irradiations with consecutive 30-sec intervals). The gingival tissues were protected with a gingival barrier of flowable composite resin (Top Dam, FGM, Joinville, SC, Brazil) light-cured for 20 sec (Valo, Ultradent, South Jordan, UT, United States). The violet LED device (MMOptics, São Carlos, SP, Brazil) was permanently positioned 8 mm away from the arches throughout the irradiation cycles. The teeth were kept hydrated with moist gauze during the intervals. The protocol was repeated for eight sessions at 4-day intervals.

(CP or LED/CP): The gingival tissue was protected with a barrier as previously described. The 37% CP gel (FGM, Joinville, SC, Brazil) was applied directly on the teeth's buccal surface. The CP gel was applied for 30 minutes without refreshing and either combined with the irradiation of the violet LED light as described above (LED/CP), or without the use of the light source (CP). At the end of the session, the bleaching gel was removed and rinsed from the teeth. The protocol was repeated for three sessions at 7-day intervals.

(HP or LED/HP): The thickener and 35% hydrogen peroxide (FGM, Joinville, SC, Brazil) were mixed in a container. This mixture was applied on the entire buccal surface from premolar to premolar with a micro brush after protecting the gingival tissues with a gingival barrier, as previously described. Initially, the gel showed a reddish color, changing to transparent within the first minutes. The HP gel was applied for 30 minutes without refreshing and either combined with the irradiation of the violet LED light as described above (LED/HP) or without the use of the light source (HP). The protocol was repeated for three sessions at 7-day intervals.

Table 2. Bleaching agents' composition and light source technical specification

Bleaching Agents and Light Source	Specification/Composition
Hydrogen Peroxide (HP) Whiteness HP (FGM, Joinville, SC, Brazil)	35% hydrogen peroxide, glycol, deionized water, dyes, inert filler, thickener, pH = 7.0
Carbamide Peroxide (CP) Whiteness HP (FGM, Joinville, SC, Brazil)	37% carbamide peroxide, glycol, deionized water, inert filler, neutralized Carbopol, pH = 7.0
Violet LED (LED) Bright Max Whitening – BMW (MMOptics, São Carlos, SP, Brazil)	Four light emitting diode lamps (401.82 nm = violet wavelength). Illumination area of the curved acrylic tip = 10.7 cm^2. Total power = 1.2 W. Irradiance at the position corresponding to the right upper incisor =8.0 mW/cm^2.

Colorimetric Evaluation

A digital spectrophotometer (Easy Shade, Vita Zahnfabrik, Bad Säckingen, Germany) evaluated the upper right canine color. A custom-made silicon barrier (Zhermak, Kouigo, Italy) of each patient's superior arch was obtained. A hole in the cervical/middle region of the upper right canine standardized the position of the spectrophotometer's tip for readings on the buccal enamel surface [28].

The baseline/initial L* (luminosity: black - / white +), a* (red + / green -), b* (yellow + / blue -), H (hue), and C (chroma) values were recorded after dental prophylaxis and before the first bleaching session (T_0) [25], and after 6 (T_{6m}) and 12 (T_{12m}) months from the last bleaching application. The patients were not submitted to dental prophylaxis at T_{6m} and T_{12m}, but they were required to brush their teeth before the follow-up sessions. During the appointment intervals, the patients were directed to not brush their teeth with whitening toothpaste. The coordinates (L*, a*, b*) were recorded and used to calculate objective color change, which was the primary outcome of the research. ΔE_{ab} and ΔE_{00} (color change) calculations were performed using the CIELab and CIEDE2000 formula, respectively, as follows [26]:

$$\Delta E_{ab} = \sqrt{\Delta(L)^2 + \Delta(a)^2 + \Delta(b)^2}$$

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + RT.\left(\frac{\Delta C'}{K_C S_C}\right).\left(\frac{\Delta H'}{K_H S_H}\right)}$$

The S_L, S_C and S_H are weighting functions that adjust the final color change in the location of L*, a* and b* coordinates. K represents parametric correction factors, and

R is a rotation function that establishes interaction among hue and chroma differences in the blue area. Two Δ values were obtained considering two time-points ($[T_{6m} – T_0]$ and $[T_{12m} – T_0]$). The 50:50% perceptibility threshold (PT) for the adopted values were 1.2 ΔE_{ab} and 0.8 ΔE_{00} units [31].

Moreover, Δ values considering the same time points above were calculated for the whiteness index for dentistry (ΔWI_D), based on the CIELab system [27]:

$$WI_D = 0.55L^* - 2.32a^* - 1.100b^*$$

The 50:50% perceptibility threshold for the whiteness index change (WPT) was considered 0.61 ΔWI_D units [32]. Finally, a visual shade guide (ΔSGU – Vita Zahnfabrik, Bad Säckingen, Germany) evaluated the subjective color change of the upper right canine [33]. The tabs of the shade guide system were sorted in terms of lightness values. According to Table 3, the numbers 1 through 16 were assigned to each value in a decreasing order. The numbers recorded in each appointment were used to calculate the subjective color change. The subjective assessment considered the same time intervals, and the evaluator was previously calibrated and blinded in terms of which group each patient belonged.

Table 3. Numeric scores of VITA Classical shade guide in decreasing order of value

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
B1	A1	B2	D2	A2	C1	C2	D4	A3	D3	B3	A3.5	B4	C3	A4	C4

Statistical Analyses

The normality and the equal variances of the data obtained in the objective colorimetric evaluation were explored using the Shapiro-Wilk and Levene tests (SPSS 23, IBM, Chicago, IL, United States). The data attending both the normality and equal variance assumptions ($p>0.05$) were submitted to one-way ANOVA and Tukey's test. Because the normality distribution of the ΔE_{ab} $[T_{6m} – T_0]$ and ΔWI_D $[T_{12m} – T_0]$ was confirmed, but the equality of variance assumption failed, data were assessed using Welch's ANOVA, followed by the *post-hoc* Games-Howell test. The data from the subjective color change (ΔSGU) were analyzed using the non-parametric Kruskal-Wallis and Dunn multiple comparisons' tests. The significance level was set at 5%.

RESULTS

The bleaching procedures and the 14-day follow-up appointments occurred in 2018 [25]. The 6-month and 1-year follow-ups assessed the long-term efficacy of the bleaching protocols during 2019. The flow of the patients throughout the clinical trial and the drop-off numbers per intervention group are illustrated in Fig. 1. Table 4 presents the demographic data of the volunteers.

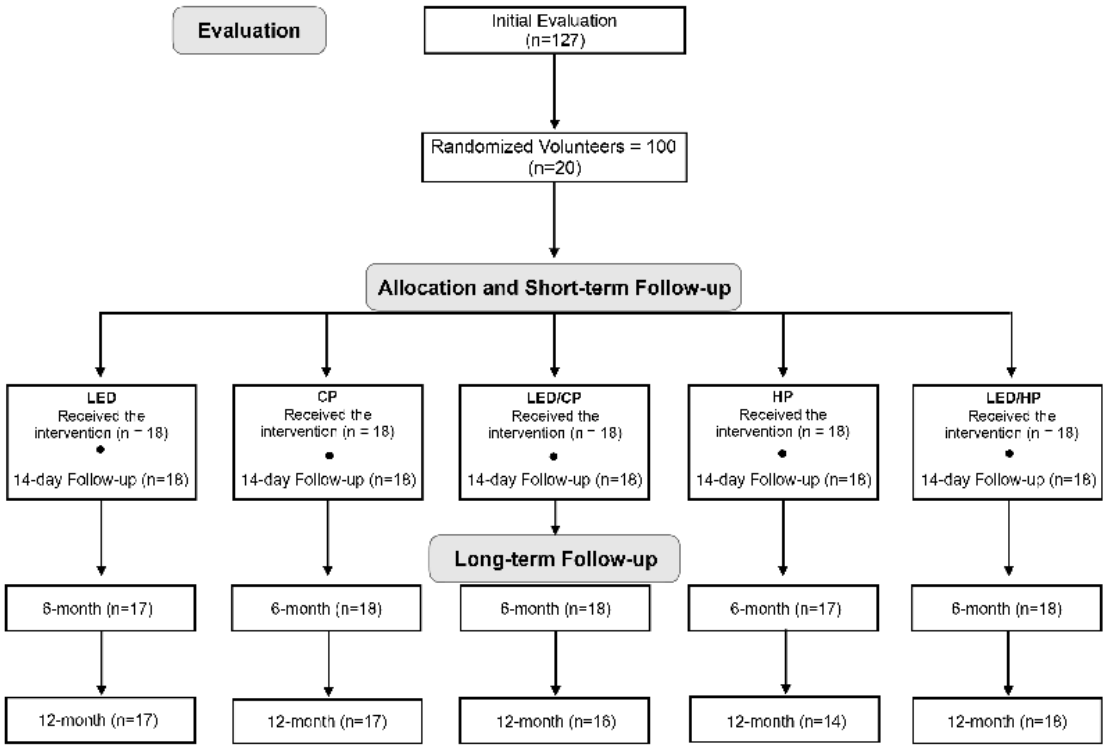

Fig. 1. Flow chart including the long-term evaluation of the volunteers.

Table 4. Demographic information of the selected patients per group as well as in an overall perspective

	AGE (Years)	GENDER	ETHINICITY
LED	22.4 (5.7)	M (30.0%); F (70.0%)	WT (90.0%); BK (10.0%); IND (0.0%); AS (0.0%)
CP	20.0 (2.3)	M (25.0%); F (75.0%)	WT (90.0%); BK (0.0%); IND (5.0%); AS (5.0%)
LED/CP	21.6 (2.4)	M (40.0%); F (50.0%)	WT (90.0%); BK (5.0%); IND (5.0%); AS (0.0%)
HP	21.7 (5.5)	M (40.0%); F (60.0%)	WT (90.0%); BK (5.0%); IND (0.0%); AS (5.0%)
LED/HP	21.2 (2.3)	M (30.0%); F (70.0%)	WT (85.0%); BK (5.0%); IND (0.0%); AS (10.0%)
Total	21.4 (4.1)	M (33.0%); F (67.0%)	WT (89%); BK (5.0%); IND (2.0%); AS (4.0%)

Abbreviations: M: male; F: female; WT: white; BK: black; IND: indigenous; AS: Asian

The observed *post-hoc* power values were above 0.95 for all the outcomes evaluated. Table 5 and Figs. 2 and 3 display the mean objective (ΔE_{ab} and ΔE_{00}) and median subjective (ΔSGU) color change values 6 and 12 months from each protocol. After six months ($T_{6m} - T_0$), violet LED exhibited the lowest ΔE_{ab} among the groups ($p<0.001$), with no color differences compared to CP (ΔE_{00} and ΔSGU; $p >0.05$). No differences were observed among CP, LED/CP and HP according to the objective (ΔE_{ab} and ΔE_{00}) and subjective parameters (ΔSGU) ($p>0.05$). LED/HP exhibited the highest ΔE_{ab} and ΔE_{00}, but its ΔSGU was comparable to those of LED/CP and HP.

Table 5. Mean and standard deviation values of the objective (ΔE_{ab} and ΔE_{00}) and median and interquartile range values of the subjective (ΔSGU) color changes after 6 and 12 months from the last bleaching session

	ΔE_{ab} ($T_{6m} - T_0$)	ΔE_{ab} - ($T_{12m} - T_0$)
LED	4.1 (1.9) ^{C}	4.9 (2.4) ^{C}
CP	7.1 (2.9) ^{B}	5.5 (3.0) ^{C}
LED/CP	8.7 (2.9) ^{B}	8.7 (2.9) ^{B}
HP	10.2 (4.3) ^{B}	10.3 (5.4) ^{AB}
LED/HP	14.3 (2.8) ^{A}	14.0 (3.3) ^{A}
	ΔE_{00} - ($T_{6m} - T_0$)	ΔE_{00} - ($T_{12m} - T_0$)
LED	3.1 (1.2) ^{C}	3.1 (1.9) ^{C}
CP	4.1 (1.8) ^{BC}	3.0 (1.7) ^{C}
LED/CP	4.8 (1.0) ^{B}	5.1 (2.0) ^{B}
HP	5.3 (2.5) ^{B}	5.2 (2.5) ^{B}
LED/HP	8.5 (1.9) ^{A}	8.4 (2.3) ^{A}
	ΔSGU - ($T_{6m} - T_0$)	ΔSGU - ($T_{12m} - T_0$)
LED	0.0 (5.7) ^{C}	0.0 (1.7) ^{C}
CP	3.0 (7.0) ^{BC}	3.0 (5.5) ^{BC}
LED/CP	6.0 (5.0) ^{AB}	5.5 (6.0) ^{AB}
HP	7.0 (3.0) ^{AB}	7.0 (4.0) ^{AB}
LED/HP	8.0 (3.0) ^{A}	7.0 (2.0) ^{A}

Means and medians followed by different letters statistically differ at 5%.

The uppercase letters compare the different bleaching protocols within the same period of evaluation

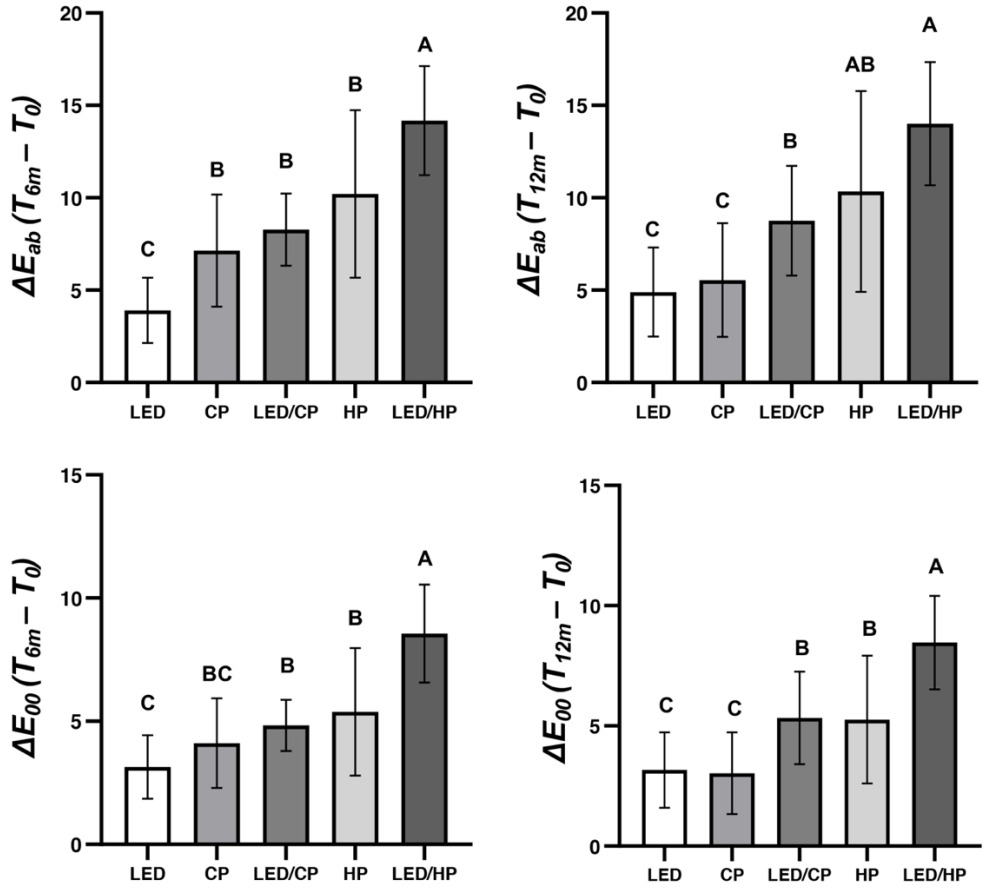

Fig. 2. Mean and standard deviation values of the objective color changes (ΔE_{ab} and ΔE_{00}) after 6 and 12 months from the last bleaching session. Means followed by different letters statistically differ at 5%. The uppercase letters compare different bleaching protocols within the same period of evaluation

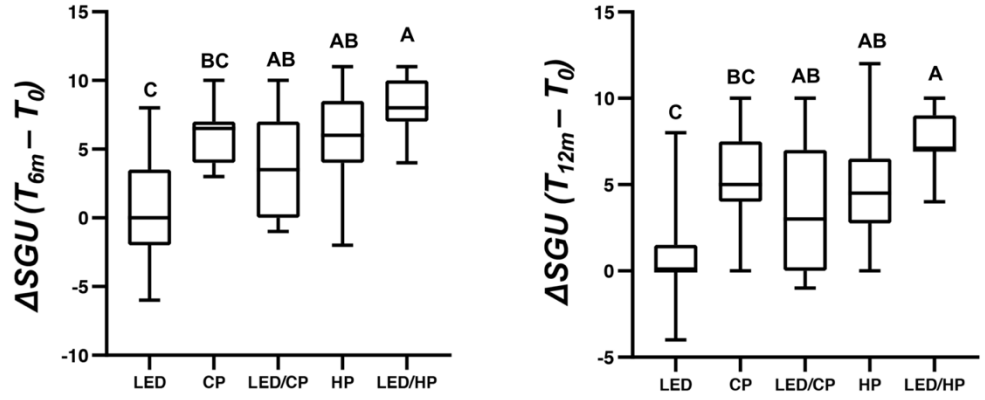

Fig. 3. Box plot of the subjective color changes (ΔSGU) showing the median, quartile ranges, minimum and maximum values after 6 and 12 months from the last bleaching session. Medians followed by different letters statistically differ at 5%. The uppercase letters compare different bleaching protocols within the same period of evaluation

After twelve months ($T_{12m} – T_0$), LED and CP groups exhibited the lowest ΔE_{ab} and ΔE_{00} ($p<0.001$). CP, LED/CP and HP exhibited no differences in terms of ΔSGU ($p>0.05$). LED/CP presented significantly higher ΔE_{ab} and ΔE_{00} than that obtained with the CP group. LED/HP exhibited the highest color changes among the groups according to ΔE_{00} ($p<0001$), but with no statistical differences to HP (ΔE_{ab} and ΔSGU) and LED/CP (ΔSGU).

All the groups presented mean ΔE_{ab} and ΔE_{00} values above the 50:50% perceptibility threshold. The differences among ΔSGU mean values presented the same statistical pattern over time, showing that LED and CP groups did not present statistical differences independently of the evaluation time. Violet LED did not increase the ΔSGU of the CP and HP gels (LED/HP=HP; LED/CP=CP).

Table 6 and Fig. 4 depict the mean ΔWI_D values after 6 and 12 months from bleaching. The LED group presented the lowest means ($p<0.05$) within separate evaluation times. The irradiation of the CP and HP gels with the LED did not significantly increase the ΔWI_D means (LED/CP=CP; LED/HP=HP) within each evaluation time. LED/CP and HP showed no statistical differences in terms of the whiteness index, independently of the time point ($p>0.05$). All the ΔWI_D means were above the 50:50% perceptibility threshold.

Table 6. Mean and standard deviation values of the whiteness index for dentistry changes (ΔWI_D) after 6 and 12 months from the last bleaching session

	ΔWI_D - ($T_{6m} – T_0$)	ΔWI_D - ($T_{12m} – T_0$)
LED	0.8 (2.6) C	1.3 (2.6) C
CP	9.6 (6.3) B	8.7 (6.8) B
LED/CP	10.9 (5.5) B	9.1 (4.8) B
HP	15.2 (8.8) AB	14.7 (12.4) AB
LED/HP	19.5 (5.4) A	19.0 (4.5) A

Means followed by different letters statistically differ at 5%. The uppercase letters compare the different bleaching protocols within the same period of evaluation

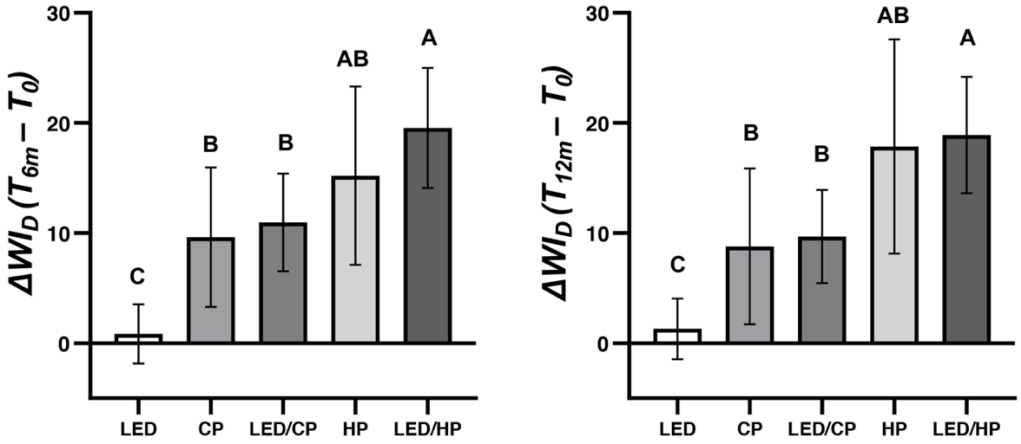

Fig. 4. Mean and standard deviation values of the whiteness index for dentistry changes (WI_D) after 6 and 12 months from the last bleaching session. Means followed by different letters statistically differ at 5%. The uppercase letters compare the different bleaching protocols within the same period of evaluation

DISCUSSION

Although there are few available clinical trials on the efficacy of violet LED for in-office bleaching [23-25], those studies demonstrated only the short-term effects on the colorimetric outcomes. The 6- and 12-month follow-ups of this study demonstrated that the violet LED alone produced color and whiteness changes significantly inferior to the HP groups. However, LED alone and CP exhibited no statistical differences concerning the ΔE_{ab} and ΔE_{00} at the 12-month evaluation. Therefore, the first null hypothesis was rejected because the violet LED alone promoted color change comparable to those of the peroxide agents. It is possible to observe that the objective differences (ΔE_{ab}) between the LED and CP groups were maintained up to the 6-month follow-up. Nevertheless, the 12-month evaluation suggested a color rebound in the CP group, which could have favored the similarity among LED and CP. Interestingly, the subjective color change (ΔSGU) revealed no differences between these two groups throughout the study.

In this context, the discrepancies among colorimetric outcomes could be credited to the method of data collection itself. The ΔSGU is considered a subjective method that lighting, age, gender, and eye fatigue might directly impact the decision of the teeth's value [34]. On the other hand, the ΔE_{00}, ΔE_{ab}, and ΔWI_D data are calculated based upon CIELab coordinates collected from precise instruments such as spectrophotometers. This equipment provides the coordinate values by means of a visible spectral reflectance process [35]. Even though subjective color determination is widely used because the visual shade guide is cost-effective, previous studies pointed out that the objective evaluations are more accurate than visual shade tabs [35,36].

Researchers hypothesized that the mechanism of the action of violet LED alone is based on the interaction of the visible wavelength (approximately 405 nm) with the extrinsic staining adhered to the surface of buccal enamel [18, 21]. Indeed, *in vitro* studies showed that the irradiation of artificially stained teeth by violet LED resulted in medium to high color or whiteness changes [19, 37, 38]. However, the outcomes obtained in the present clinical scenario suggested that the light effect alone is perceptible, but less efficient than high-concentrated hydrogen peroxide. Thus, the fact that patients exhibited teeth with lower staining and were submitted to prophylaxis before bleaching might have impacted the outcomes.

Since the whiteness index for dentistry calculation takes into account the CIELab coordinates [27], it makes sense that the WI_D changes promoted by violet LED were significantly lower than the CP and HP groups. The WI_D evaluation indicates changes in the teeth's spectral behavior that migrate to high lightness and low saturation [27]. Also, this index performs a greater correlation with visual color perception. Based on the ΔWI_D presented in this study, it is possible to infer that the impact of the light itself on the whiteness of teeth was significantly lower than that of peroxide-based agents.

The color change calculation based on the CIEDE2000 system is an important data that was also not used in the previous clinical trials on the violet LED in-office bleaching [23,24,25]. The differences among the ΔE_{ab} and ΔE_{00} calculations rely on the weighting functions that adjust the final ΔE_{00} in the location of the L*, a* and b* coordinates. Applying the lightness, hue, and chroma, CIEDE2000 corrects the interaction of hue and chroma in the b* coordinate. It also alters the low influence of the a* coordinate, which is important only for colors with low chroma [39]. A recent review from Paravina et al. (2019) displayed the individual ΔE_{ab} and ΔE_{00} values compatible with visual perceptibility as well as with excellent efficacy of bleaching [40]. Therefore, the authors of this study believed that the inclusion of these three objective parameters would guarantee a more accurate and broader discussion.

The violet LED irradiation increased the ΔE_{ab} and ΔE_{00} of the CP gel, but only at the 12-month evaluation. Thus, the second null hypothesis that bleaching gels irradiated by violet LED would not present higher changes than CP or HP alone was rejected. The decrease in the color change of the CP group (without light) after 12 months could be credited for the lower decomposition of the CP gel into hydrogen peroxide [6]. However, a 6% HP gel, which presents lower total hydrogen peroxide concentration than 37% CP, resulted in stable color change after one year [41]. Therefore, the presence of urea in the CP gel might have decreased the CP by-products (without LED irradiation) interaction in the dentin. Also, the characteristics and habits of each patient cannot be ruled out, because the patients' habits, such as the consumption of dark beverages or even toothbrushing, influence the color rebound [42].

On the other hand, the more stable LED/CP outcomes might be a result of the synergistic effect of the CP itself and the photolytic activity of the violet light [9]. Even

though previous clinical trials concluded that the painful sensation during bleaching was lower for the CP protocol, CP did not attain the chromatic changes as observed for HP [7,25]. Vaez et al. (2019) showed that the humidification with a damp gauze of enamel prior to the 37% CP application enhanced the efficacy of the in-office bleaching protocol [43]. Thus, activation methods could be appropriate to increase the 37% CP bleaching outcomes. LED/CP presented a color change significantly higher than CP only at the 12-month evaluation, but its similarity with HP was observed independent of the evaluation time. Therefore, the third null hypothesis that LED/CP and HP groups' changes would not be similar after 12 months was rejected. Clinically, this observation for LED/CP could be extrapolated to a stable and high-efficient [40] bleaching protocol with lower levels of tooth sensitivity [25] in comparison to 35% HP.

The significantly higher ΔE_{ab} and ΔE_{00} results of LED/HP compared to the HP protocol observed at the 6-month follow-up were only replicated for ΔE_{00} at the 12-month follow-up. An important limitation regarding these results was the different number of patients able to return to the last appointment in each group. The drop-off was justified because patients moved to long-distance cities. The highest drop-out rate in the HP group (n=4) at T_{12m} could have been responsible for the increase in the standard deviation, not resulting in statistical difference between the groups in the ΔE_{ab} evaluation. However, the CIEDE2000 system displayed a higher color change for LED/HP even under such circumstances. Regardless of the increase detected in ΔE_{00} for LED/HP, it is worth mentioning that the intensity of tooth sensitivity for this group during bleaching was overall higher in comparison to HP [25]. Also, the HP and LED/CP protocols exhibited excellent bleaching efficacy [39], thereby questioning the necessity of irradiating the HP gel.

It is important to highlight that the colorimetric analyses herein were only performed using the upper right canine. The decision to use the upper canine was based upon the fact that the chromophores are located in the dentin [6]. Also, a previous study in the literature showed that the esthetic outcomes on thicker teeth tend to saturate later than in thinner teeth, i.e. the upper central incisor [44]. However, further studies could attempt to evaluate if the bleaching outcomes with the present protocols would be different in other dental elements.

Since all the patients reported absence of tooth sensitivity symptoms after one week from the last bleaching appointment, no tooth sensitivity data was added to this study. No complaints regarding tooth sensitivity were reported at either the 6- and 12-month follow-ups. Also, further studies could evaluate other application times of the bleaching gels, as the present clinical trial applied the protocols recommended by the light manufacturer and published in previous studies [19,20]. Following the American Dental Association (ADA) guidelines, effective and safe performance of bleaching in patients is dependent on an appropriate standard exam and the correct diagnosis of the dental discoloration prior to treatment [45].

To summarize, the colorimetric evaluation over time of patients showed that the color and whiteness changes caused by the violet LED alone is perceptible. However, this protocol did not translate into the high levels of bleaching efficacy and did require a longer treatment time. In other words, patients would be submitted to 8 sessions of violet LED bleaching without reaching the esthetic outcomes observed for peroxide-driven bleaching. On the other hand, the violet LED irradiation of CP promoted stable colorimetric changes and exhibited long-term similar efficacy to HP. At the 12-month follow-up, the ΔE_{00} was the only colorimetric parameter showing significant increase in efficacy for LED/HP.

CONCLUSIONS

Within the limitations of this study, the possible conclusions could be drawn:

- The use of violet LED alone (without bleaching gels) resulted in perceptible long-term bleaching outcomes, but its efficacy was significantly lower than LED/CP and HP-containing protocols;
- The increase in the long-term efficacy of CP and HP gels irradiated with violet LED was dependent on the evaluation time and the colorimetric system. The combination of violet LED with gels tended to increase the long-term color change (ΔE_{ab} and ΔE_{00}) outcomes, but not the ΔWI_D results; and
- LED/CP reached the efficacy (ΔE_{ab}, ΔE_{00} and ΔWI_D) of the HP protocol even after 12 months after the bleaching procedures.

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2.3 Artigo 3. Novel experimental in-office bleaching gels containing co-doped titanium dioxide nanoparticles

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ABSTRACT

The present study reported on the development and testing of novel bleaching agents containing co-doped metaloxide nanoparticles (NP; 0%, 5%, 10% v/w) and hydrogen peroxide (HP, 0%, 6%, 15% and 35%). Bovine blocks (n=200, A=36mm^2) were obtained and randomly distributed into experimental groups (n=10/group). NPs were incorporated into gels before bleaching (3 sessions, 7 days apart, 30 min/session, irradiated with violet light - LT). Color changes (ΔE_{00}, ΔWI_D), mineral content (CO_3^2, PO_4^3) and topography were assessed (spectrophotometer, ATR-FTIR and AFM) before and after bleaching procedures (14 days). Metabolic status and 3-dimmensional components of non-disrupted *Streptococcus mutans* biofilms were investigated using a multimode reader and confocal microscopy. Results indicate that ΔE_{00} and ΔWI_D significantly increased with NPs concentrations and LT. Enamel's mineral ratio was adversely impacted by HP but alterations were less pronounced when using NP-containing gels. Enamel's topography was not damaged by bleaching protocols tested. Bioluminescence results shown that bleaching protocols don't render latent antibacterial properties to enamel and confocal microscopy results demonstrated that 3-dimmentional distribution of components was affected by protocols. The nanotechnology proposed improved the bleaching efficacy of experimental materials independent of hydrogen peroxide or irradiation, did not adversely impact enamel's surface properties nor its chemical content.

Keywords: Tooth Bleaching, Hydrogen Peroxide, Light Irradiation, Nanoparticles

INTRODUCTION

In-office power bleaching (IPB) is considered an ultraconservative and minimally invasive treatment capable of resolving dental discolorations (low to moderate) in as short as one clinical session [1]. The IPB treatment typically involves three clinical sessions (45 min each; 7-days apart) using hydrogen peroxide-containing bleaching gels (HP, 35% to 45%) in combination or not with visible light irradiation [2] to promote the attainment of immediate esthetic outcomes. IPB's underlying mechanism of action revolves around the generation of reactive oxygen species (ROS). Upon generation, these short-lived and highly-reactive free radicals must be efficiently transported from the gel to the dentin-enamel junction (DEJ). Once at the DEJ, free radicals will then break conjugated double bonds present in large organic molecules (chromophores) through a non-specific oxidative process [3].

Even though several reports have demonstrated the bleaching efficacy of IPB, [4-6] other studies have indicated that the utilization of these highly caustic bleaching agents may result in the occurrence of adverse effects (short- and long-term) including irreversible changes in enamel topography [7] and chemical make-up [8-10], decreased surface microhardness [11], increased surface roughness [12], diminished bond strength [13] and reduced fracture resistance [14]. From the clinical standpoint, the most prevalent adverse effect reported by patients and clinicians is mild to severe dentin hypersensitivity (DH) [4, 15, 16]. According to previous studies, there is a strong and positive correlation among dentin hypersensitivity, HP concentration and pulpal cytotoxicity [17, 18], where the higher the HP concentration, the stronger the dentin hypersensitivity [16] and more durable are the effects.

In this critical context, several research groups have tried to overcome limitations described by adding calcium or fluorine ions in the formulation of highly concentrated bleaching gels. Even though results reported have demonstrated that adverse effects, such as decreased enamel microhardness and rougher surfaces were less pronounced with the utilization of calcium- or fluorine-containing gels [8], subsequent studies have shown that promising results initially reported were limited to the outermost layers of enamel, and did not prevent the loss of minerals at subsurface levels, thereby restricting the therapeutic effect of novel formulations proposed [9]. Follow-up studies investigated

the efficacy of experimental protocols modulated by low-concentrated bleaching gels (6-15%) and near-UVA wavelengths (405 ± 15 nm), as an alternative approach to reduce the incidence of dentin hypersensitivity while trying to achieve desirable whitening outcomes [19-21]. Even though the utilization of low-concentrated bleaching gels resulted in lower incidences of DH, the bleaching efficacies reported (in terms of ΔE and whitening index [WI]) were considered poor because outcomes were much less intense and durable, as compared to those attained with gels containing high HP concentrations.

Recent approaches focused on the incorporation of metaloxides, such as titanium dioxide (TiO_2, P25 Degussa) and nitrogen-doped titanium dioxide (N_TiO_2) nanoparticles, into the formulation of commercially-available bleaching gels containing high HP concentrations [22-24]. In theory, the incorporation of these semiconductors would improve the dissociation of HP into ROS, by a photo-physical process, where photons are converted into thermal energy. However, despite the theoretical feasibility of the process, experimental bleaching gels containing varying concentrations of metaloxide nanoparticles were demonstrated to be clinically ineffective when compared to unaltered gels containing HP (either 15% or 35%) [22, 23]. These unexpected findings are believed to have precipitated from fast and spontaneous dissociation processes that take place when HP is exposed to metaloxides, and from other contributing factors such as limited wettability and high viscosity.

A recent study reported on the successful fabrication of N_TiO_2 (6-15 nm) using highly controllable, reproducible, and green solvothermal reactions [25]. In that study, nanoparticles synthesized were incorporated into commercially-available dental adhesive resins (OptiBond Solo Plus, Kerr Corp.) with the objective of imparting non-leaching antibacterial and biomimetic functionalities to the parental polymer. According to Huo et al. [26], the synthesis route reported by Esteban Florez et al. [25] results in the attainment of pure and crystalline TiO_2 nanoparticles (anatase phase) that are electron deficient, display high levels of nitrogen doping, have well-defined pore structure, large surface areas, facilitate the generation of electron-hole pairs, and are capable of efficiently absorbing visible wavelengths (400 to 700 nm) while generating significant amounts of perhydroxyl ($HO_2^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals [25], which are long-lived species of oxygen.

Follow up studies from the same research group demonstrated the successful solvothermal synthesis of TiO_2 nanoparticles that were co-doped with either nitrogen and fluorine (NF_TiO_2) or nitrogen and silver (NAg_TiO_2), functionalized into OptiBond Solo Plus, and tested for antibacterial properties (in dark and light irradiated conditions) against *Streptococcus mutans* using a newly developed and optimized high throughput bioluminescence assay [27, 28]. According to results reported, experimental materials containing 30% of either NF_TiO_2 or NAg_TiO_2 displayed antibacterial behaviors that were comparable to those attained with Clearfil SE Protect (Kuraray Co.; fluoride-releasing material) independently of light irradiation conditions [27]. These findings have not only indicated that the nanotechnology reported has a strong potential to be translated into commercial products capable of sustaining long-term antibacterial properties, but the promising antibacterial effects observed in the absence of light corroborate the findings reported by Huo et al. [26] that nanoparticles synthesized through solvothermal processes are capable of generating long-lived species of oxygen.

Based on that premise and considering that fluorine is one of the most reactive chemical elements known to man, our research group decided to functionalize NF_TiO_2 (NP) into experimental bleaching gels containing HP (6%, 15% or 35%) and determine the effects of nanoparticles' concentration (0%, 5% and 10%) and violet light irradiation on bleaching efficacy, bovine enamel chemical make-up and surface topography. Additional analyses were focused on revealing how experimental bleaching protocols affect the metabolism and the components of single-species biofilms using a minimally invasive, real-time and high throughput bioluminescence assay and a concurrent staining technique along with confocal laser scanning microscopy, respectively. The null hypotheses were that the incorporation of NP would not significantly affect the (i) the bleaching efficacy and (ii) the chemical make-up of enamel bleached with the experimental bleaching gels. In addition, it was hypothesized that the incorporation of NP would not (iii) avoid the growth of biofilm on the bleached enamel surface.

MATERIALS AND METHODS

Experimental Design

The specimens described in section 2.2 below (n=200; n=10/group) were randomly allocated according to the study factors:

Bleaching Agent:

- 0% hydrogen peroxide (0% HP)
- 6% hydrogen peroxide (6% HP)
- 15% hydrogen peroxide (15% HP)
- 35% hydrogen peroxide (35% HP)

NF_TiO_2 Concentration (v/w):

- 0% NP
- 5% NP
- 10% NP

Light Activation:

- Dark conditions
- Visible light (LT)

Analyses of color (ΔE_{00}, ΔWI_D), pH of the experimental gels, mineral composition (carbonate:phosphate ratio), surface topography of enamel and microbiological activity evaluation were conducted. Initial surface analyses were performed before bleaching – baseline (T_0). Experimental bleaching protocols consisted of three sessions (T_1 = first bleaching session, T_2 = second bleaching session, T_3 = third bleaching session). Analyses following the bleaching protocols were carried out 14 days after (T_4) the third bleaching session (T_3).

Specimen Preparation and experimental groups

Squared shaped specimens (Enamel-dentin blocks; Area = 36.0 mm^2, thickness = 3.0 mm) were obtained from the central buccal area of bovine crowns as described in previous studies [10, 29]. The blocks were polished using a rotary polisher (Arotec, São Paulo, SP, Brazil) and abrasive disks (600- and 1,200-Grit, Norton Saint-Gobain, Guarulhos, SP, Brazil) and finished using polishing cloths (3M Brazil, Sumaré, SP, Brazil) with diamond suspensions (1 μm, 0.50 μm and 0.25 μm, Erios, São Paulo, SP, Brazil).

Prepared specimens were subjected to Knoop microhardness testing (50.0 g load, 5 s/indentation, 3 indentations/specimen, 100 μm apart; Future Tech FM-ARS, Tokyo, Japan). [30] Specimens (n = 200; 10/group) with standardized microhardness (296.07 kgf/mm^2 ± 29.60) were randomly distributed and submitted to bleaching with hydrogen peroxide (HP; 0%, 6%, 15% and 35%) experimental gels, containing NF_TiO_2 nanoparticles (NP; 0%, 5% and 10%), and violet light irradiation (LT; with or without):

- G1 – No treatment (control group);
- G2 – LT,
- G3 – HP6;
- G4 – HP6 + LT;
- G5 – HP15;
- G6 – HP15 + LT;
- G7 – HP35;
- G8 – HP35 + LT;
- G9 – HP6 + 5%NP;
- G10 - HP6 + 5%NP + LT;
- G11 – HP15 + 5%NP;
- G12 – HP15 + 5%NP + LT;
- G13 – HP35 + 5%NP;
- G14 – HP35 + 5%NP + LT;
- G15 – HP6 + 10%NP;
- G16 – HP6 + 10%NP + LT;
- G17 – HP15 + 10%NP;
- G18 – HP15 + 10%NP + LT;
- G19 – HP35 + 10%NP;
- G20 – HP35 + 10%NP + LT.

Nanoparticles' Synthesis

A detailed description of the synthesis of NF_TiO_2 nanoparticles has been reported in previous publications [25, 27, 28]. A solution of 1.7 g of $Ti(OBu)_4$ (Aldrich, 97%), 4.6 g C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, USA), 6.8 g $C_{18}H_{35}NH_2$ (Aldrich,

70%), 7.1 g $C_{18}H_{34}O_2$ (Aldrich, 90%) and 5% of NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar) was prepared and mixed with an ethanol-water solution (4%, 18-Milli-Q; total weight = 13.10 g). Solutions prepared were transparent before mixing, however, the final solution clouded instantaneously after mixing due to hydrolysis and some micelle formation. The final solution was placed into a high-pressure reaction vessel (Borosilicate Glass-lined; Paar Series 4593, Bench Top Reactor System, Moline, Il, USA), reacted (180 °C, 24 hours, 15 psi), and stirred via external shaft coupled to a turbine impeller (280 rpm). At the end of the 24-hour cycle, the solution was removed from the reaction vessel, and transferred to a 50 mL falcon tube with a certain amount of ethanol (200-proof, Decon Labs, King of Prussia, PA, USA). The solution was centrifuged for 15 min at 8,000 rpm. This procedure was repeated two additional times, using 20 mL of ethanol.

Polymer Synthesis and Incorporation of NPs

Experimental bleaching gels were formulated in our laboratory by mixing a commercially-available hydrophilic polymer (12.5 g, Carbomer 940 NF, Spectrum, Gardena, CA) with an aqueous solution (distilled, 400 mL, pH = 11) containing KOH (60%, 20 mL) using a planetary and orbital stand-alone mixer (1 cycle at 2,000 rpm for 2 minutes, two additional cycles at 2,500 rpm for 3 minutes each; Speed Mixer, DAC 400.1 FVZ, FlackTek Inc, Laudrum, SC, USA). Immediately after mixing, the resulting polymer (pH ~ 6) was observed to be transparent and free of any undissolved polymer (white agglomerates). The experimental polymer was then stored in a black container for at least 24 hours (refrigerator, 8^{o}C).

Two aliquots (1 mL and 2 mL, respectively) of nitrogen and fluorine co-doped titanium dioxide nanoparticles (NF_TiO_2, ~ 40 mg/mL) suspended in ethanol (described in section *2.2 Nanoparticles Synthesis*) were placed in individual plastic tubes and were centrifuged (8,000 rpm, 5 min) in preparation for polymer incorporation procedures. Ethanol-free nanoparticles were then individually mixed into 20 g of the experimental polymer to render gels containing either 5% or 10% of NP. Each nanofilled gel was then mixed at 2,450 rpm for 20 s (Speed Mixer, DAC 400.1 FVZ, FlackTek Inc, Laudrum, SC, USA). The final gel continued to be transparent and free of visible agglomerates, but its color became pale-yellow due to the successful incorporation and dispersion of NP.

Incorporation of Hydrogen Peroxide (H_2O_2)

Immediately before utilization, experimental gels (either 1 g or 1.5 g, depending on H_2O_2:polymer ratio) with or without NP (either 5% or 10%) were manually mixed (1:2 [6% or 15% H_2O_2] or 2:3 [35% H_2O_2]) with 1 mL of hydrogen peroxide following previously published protocols (Figure 1). [10, 19] The rationale for the utilization of two distinct H_2O_2:polymer ratios was based on the need to achieve comparable viscosities for all experimental materials investigated.

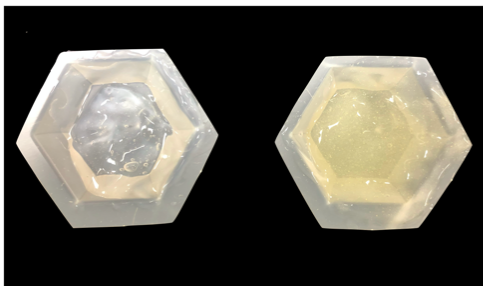

Figure 1. Appearance of experimental polymers without *(left)* or with *(right)* 10% of NP. Note that the incorporation of NPs in the concentration mentioned rendered experimental materials that were transparent, had pale-yellow color and were free of large agglomerates

Bleaching protocols

Experimental bleaching protocols investigated consisted of three sessions (T_1= first bleaching session, T_2= second bleaching session and T_3 = third bleaching session) 7-days apart. Each 30-minute session was based on a single application of the proper experimental gel (with or without nanoparticles) combined or not with continuously visible light irradiation (20 cycles of 1 min, 30 second intervals between irradiation cycles [19]; 405 ± 15 nm, 1.2 W/cm^2, emission window area = 10.7 cm^2, Bright Max Whitening, MMO, São Carlos, SP, Brazil) according to experimental groups (G1 to G20, see group descriptions in section *2.2. Specimen Preparation and experimental groups*). Figure 2 illustrates specimens subjected to dental bleaching procedures modulated by experimental bleaching gels and visible light irradiation (G14 - HP35+5%NP+LT). After each session, specimens from all groups were stored (37^{o}C, dark conditions) in artificial saliva (1.5 mM calcium chloride [$CaCl_2$], 0.9 mM sodium phosphate [NaH_2PO_4], 0.15 mM potassium chloride [KCl, pH 7.0]). After the third session (T_3), specimens were then stored in artificial saliva for 14 days using the same procedures previously described [10].

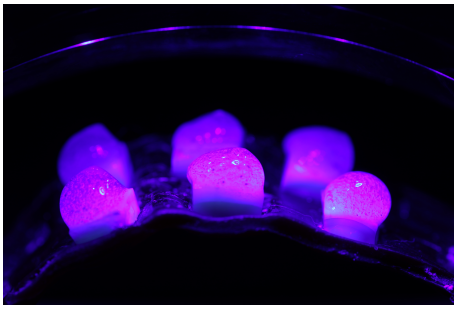

Figure 2. Specimens from G14 – HP35+5NP+LT being subjected to IPB with visible light irradiation

Objective Colorimetric Evaluation

The objective colorimetric evaluation (in terms of L*, a*, and b*) was performed before the first bleaching session (baseline, T_0) and 14 days after the last bleaching session (T_4) using a hand-held digital spectrophotometer (Vita EasyShade, VITA Zahnfabrik H. Rauter GmbH & Co. KG, Bad Sackingen, Germany). Variation of color (T_4-T_0) was determined using the formulae for ΔE_{00} (eq. 1) [31] [32] and ΔWI_D (eq. 2) [33] as follows:

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + RT \cdot \left(\frac{\Delta C'}{K_C S_C}\right) \cdot \left(\frac{\Delta H'}{K_H S_H}\right)} \quad \text{(eq. 1)}$$

$$WI_D = 0.55L^* - 2.32a^* - 1.100b^* \quad \text{(eq. 2)}$$

pH Analysis

The temporal evolution (10-minute increments, total time = 30 min) of pH was determined for experimental bleaching gels (1 g of each, with or without nanoparticles) irradiated or not with visible light using a calibrated pH meter (AB150, Accumet, Fisher-Scientific, Hampton, NH, USA) to determine the impact of pH on properties investigated. This analysis was carried out during the last bleaching session (T_3).

Mineral Content Evaluation

Infrared spectra of bovine enamel at T_0 and T_4 were acquired at three locations per specimen using a Fourier Transform Infrared spectrometer (Nicolet IS50, Thermo Fisher, Madison, WI, USA; scanning parameters: 500 - 4,500 cm^{-1}; resolution 4 cm^{-1}, 10 internal scans per spectrum/ location) coupled to a heated attenuated total reflectance (ATR) monolithic diamond crystal (Golden Gate, Specac, Fort Washington, PA, USA). A method

previously described [7, 34] was utilized to guarantee that ATR-FTIR measurements were performed exactly at the same locations in each specimen. Enamel spectra (at T_0 and T_4) from each specimen were corrected for the presence of water before being subjected to baseline correction and normalization procedures using the OMNIC software (v7.0, Madison, WI, USA). The areas under the peaks corresponding to CO_3^2 υ2 (886 cm^{-1}), PO_4^3 υ1 (996 cm^{-1}) and PO_4^3 υ2 (1,410 – 1,460 cm^{-1}) were calculated after experimental treatments (T_4). The mineral composition of enamel (in terms of carbonate:phosphate mineral ratio) was determined by integrating the areas under the curves of CO_3^2 υ2 and PO_4^3 (υ1 and υ2) [7].

Topography Assessment

An atomic force microscope (MultiMode with Nanoscope V controller, Bruker, Billerica, MA, USA) in ScanAsyst mode coupled with silicon nitride probes (aluminum-coated, triangular, radius = 2 nm, spring constant = 0.4 N/m, Bruker) was used to reveal topographical aspects of specimens (n = 1/group) at T_0 and T_4. Images (A = 625 μm^2; 512 x 512 lines) were acquired (at the same locations at T_0 and T_4) using a scan rate of 0.8 Hz. Images were then flattened before acquiring topographical parameters of interest (R_a [roughness average] and R_q [root mean square roughness]) using the Nanoscope software (v9.0, Bruker).

Metabolic status of non-disrupted biofilms

A minimally invasive, real-time, and high throughput bioluminescence assay recently reported by Esteban Florez et al. [27] determined the metabolic status of non-disrupted *Streptococcus mutans* biofilms grown on the surfaces of specimens only after being treated (T_4) with the experimental groups (n= 18/group) described in section *2.2 (Specimen Preparation and experimental groups)*. These specimens were prepared especially for this methodology. In brief, planktonic cultures of *Streptococcus mutans* (JM10) were grown overnight (16 hours) in a liquid culture medium (THY) at oral temperature. Cultures having an optical density higher than 0.900 (at 600 nm; corresponding to 6.43 e^{+12} CFU/mL) were used as inoculum to grow biofilms. *S. mutans* biofilms were then grown (24 hours, microaerophilic conditions, 37°C) on the surfaces of

sterile specimens (UV-sterilized, 254 nm, 800,000 μJ/cm^2, UVP Crosslinker, model CL-1000, UVP, Fisher Scientific, Hampton, New Hampshire, USA) using inoculated biofilm growth media (0.65x THY, 1:50 dilution, 1.0 mL/well) supplemented with sucrose (1%, w/v). After 24 hours, biofilms were immersed in 1.0 mL of fresh 1x THY + 1% (w/v) glucose recharge medium and were incubated (37°C, 1 hour) before being transferred into the wells of sterile white 24-well plates containing 1.0 mL of fresh 0.65x THY + 1% (w/v) sucrose medium. An aqueous solution (100 mM) of D-Luciferin suspended in citrate buffer (0.1 M, pH 6.0) was added by a Synergy-HT multimode plate reader (Biotek, Winooski, VE, USA) to the wells containing both the specimens and biofilms in recharge medium (2:1 ratio [v/v] inoculum to D-Luciferin). The metabolic activity of non-disrupted biofilms was assessed (in terms of RLUs) at 590 nm in 2-min increments (total of 6 minutes) after the addition of D-Luciferin.

Staining and confocal microscopy

A concurrent staining method previously reported by Khajotia et al. [35] was used to illustrate the impact of experimental bleaching treatments on the distribution of nucleic acid, proteins, and extracellular polymeric substances (EPS) biofilm components. To achieve this goal, an additional set of specimens (n = 1/group) were prepared and bleached according to the methods previously described (sections *2.2* and *2.6*). Biofilms were grown on the surfaces of sterile specimens at T_4 using the methods described in section *2.11*. After the 24-hour growth period, biofilms were washed with PBS (3x, pH 7.4, 25°C, 15 s/wash) to remove non-adherent cells. Biofilms were then concurrently stained with Alexa Fluor® 647 conjugate of Concanavalin A (Invitrogen, USA; 250 μg/mL), Syto 9 (Molecular Probes, USA; 10 μM), and Sypro Red (Invitrogen, USA; 10x). Biofilms were kept hydrated in sterile ultra-pure water and protected from light until confocal microscopy. Images of biofilms were acquired using a TCS-SP2 MP confocal laser scanning microscope (CLSM, Leica Microsystems, Inc., USA) with Ar (488 nm) and He/Ne (543 and 633 nm) lasers for the excitation of the fluorescent stains within biofilms at three different locations on each specimen's enamel surface. A 63x water immersion microscope objective lens was used. Serial optical sections were recorded from the surface of specimens to the top of biofilms at 0.6 μm intervals in the Z-axis. 3-D images of the biofilms

were generated using Volocity software (PerkinElmer, USA) to allow the visualization of the distribution of the nucleic acid (green fluorescence), proteins (red fluorescence), and EPS (blue fluorescence) components of biofilms.

Statistical Analyses

Linear Models (two- and three-way ANOVA) statistical analyses with outcomes including ΔE_{00} and ΔWI_D ($T_4 - T_0$) and the carbonate:phosphate mineral ratio (only at T_4) were fitted. The mineral ratio data were transformed into log. Factors included HP concentration (4 levels: without HP, 6% HP, 15% HP and 35% HP), NP concentration (3 levels: without NP, 5% NP and 10% NP), and LT (2 levels: with or without light). A backward model selection strategy was adopted with the full model containing all the main effects, all the two-way interactions (HP*NP, HP*LT, NP*LT), and the three-way interaction (HP*NP*light). A term was removed from the model if its p-value was less than 0.05 and the removal process started with the highest order term. In addition, RLU obtained from the metabolic status analysis of the biofilms were submitted to the general linear model procedure considering the factors group (G1-G20) and time (0, 2, 4 and 6 min) with post hoc Student-Newmans-Keuls tests. All the analyses were conducted using SAS software (version 9.3, SAS Institute, USA) at a 5% level of significance.

RESULTS

Bleaching efficacy

The findings reported in Figure 3 have demonstrated that experimental bleaching protocols modulated by gels containing 6%, 15% and 35% of H_2O_2 (without NP or LT) displayed mean values of ΔE_{00} and ΔWI_D that were higher when compared to those of the control groups (no treatment, with or without LT). The two-way interactions among the factors are displayed in Figure 4. These results have also indicated that ΔE_{00} and ΔWI_D values varied with HP concentrations ($p < 0.0001$), and bleaching outcomes could be rank ordered in terms of increasing efficacies where HP6 < HP15 < HP35, respectively. Even though a similar trend was observed when bleaching protocols were modulated by experimental bleaching gels containing 6%, 15% and 35% of HP (without NP) and visible light irradiation (405 nm ± 15 nm), ΔE_{00} and ΔWI_D values were higher than those from bleaching protocols with no light irradiation ($p < 0.0001$). The combination of HP and NP further increased the efficacy of experimental bleaching protocols, as denoted by mean ΔE_{00} and ΔWI_D values of HP6 and HP15 incorporated with 5% of NPs.

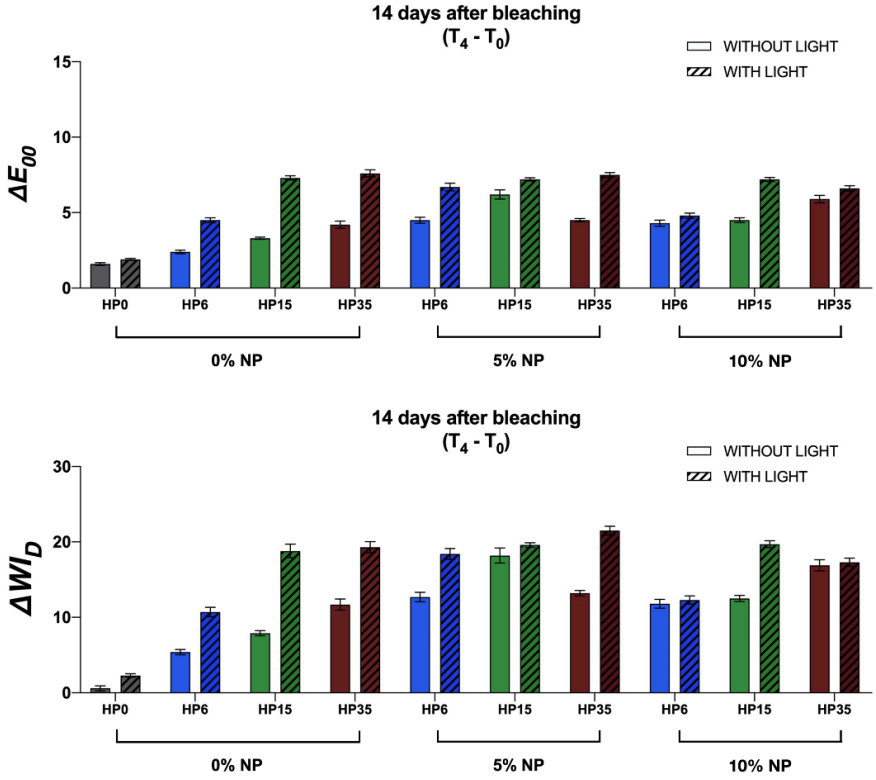

Figure 3. Mean and standard error values of ΔE_{00} and ΔWI_D that were calculated considering the coordinate values collected before (T_0) and 14 days (T_4) after the last bleaching session with 6%, 15% and 35% HP incorporated or not with NP (either 5 or 10%)

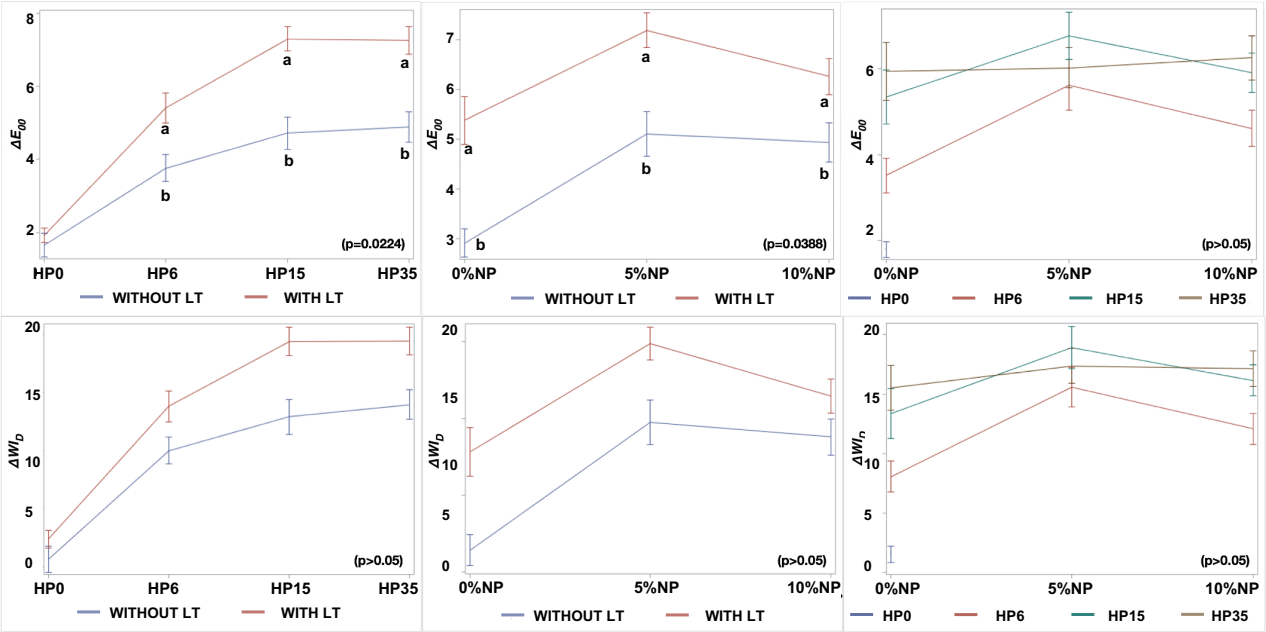

Figure 4. Marginal ΔE_{00} and ΔWI_D means and standard errors within each level of HP (0%, 6%, 15%, 35%), NP (0%, 5% and 10%), and LT (with or without) according to two-way interactions HP*LT, NP*LT and NP*HP, respectively. Distinct letters represent difference within the same HP or NP concentrations, taking into consideration a 0.05 level of significance

Analysis of pH

The graphs displayed in Figure 5 illustrate the temporal evolution of pH for experimental gels (6%, 15% and 35% HP) with or without NP (5% and 10%). It is possible to observe that experimental gels (6%, 15% and 35% HP) without the incorporation of NP displayed pH values (≅ 5.0, 6% HP + LT at 20 min) that were lower when compared to those containing NP. Such behavior was observed to be consistent throughout the observation time (at 0, 10, 20 and 30 min), The results reported have also indicated that such behavior is not influenced by visible light irradiation and that nanofilled bleaching gels displayed comparable pH values after 30 minutes.

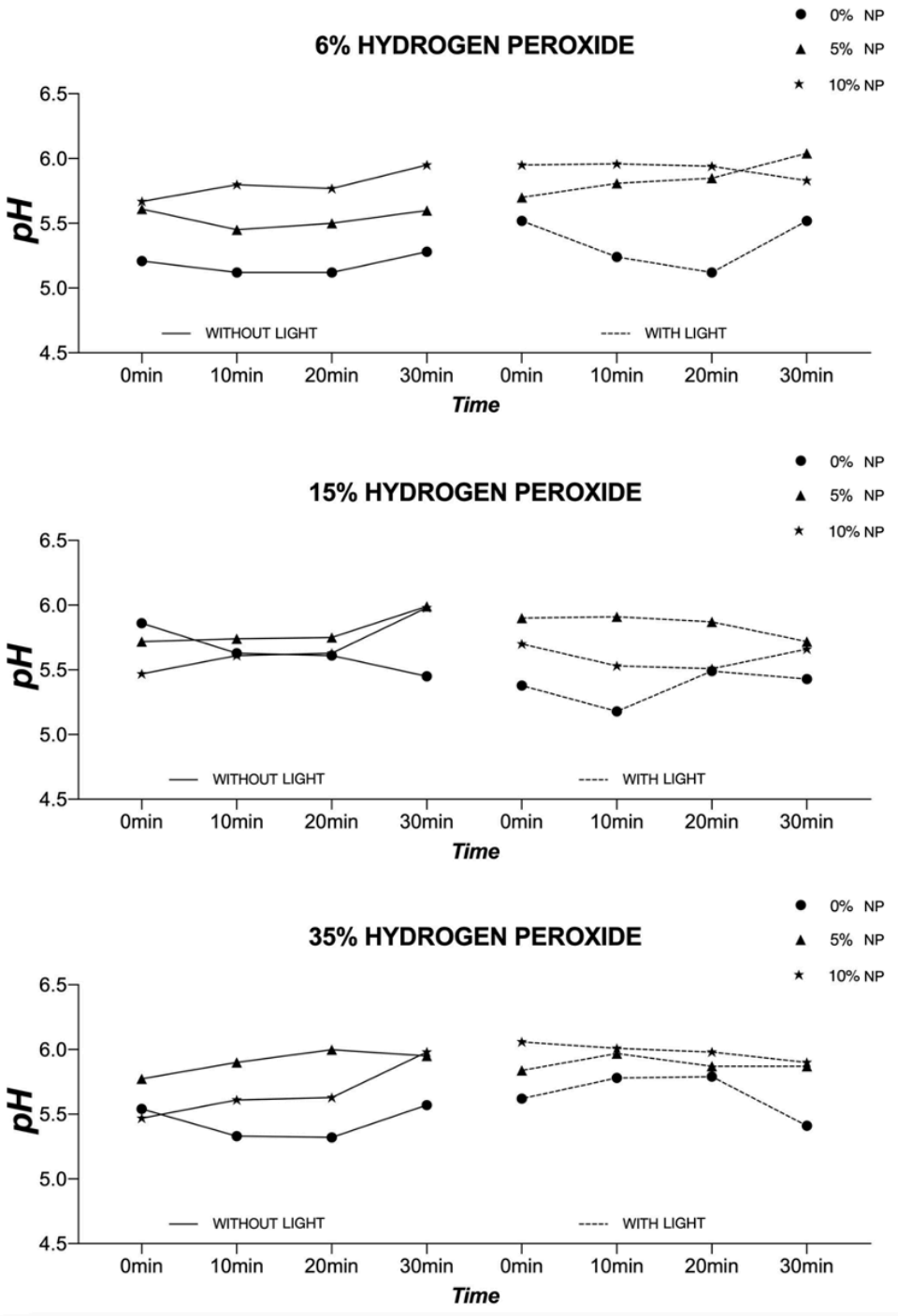

Figure 5. Temporal evolution (0, 10, 20 and 30 min) of pH values of the experimental gels (6%, 15% and 35% HP) with or without NP and LT

Mineral content evaluation

The linear models showed that only the isolated factors HP ($p < 0.0001$) and NP ($p < 0.0001$) were significant to the carbonate:phosphate mineral ratio variable. The LT factor, two-way and three-way interactions were not significant (Figure 6). Figure 7

illustrates the impact of experimental bleaching gels on the mineral ratio CO_3^2/PO_4^3 of bovine enamel. It is possible to observe a decrease in T_4 in all groups investigated where values varied from 0.14 ± 0.03 (6HP+LT) to 0.20 ± 0.05 (35HP+NP10%+LT) compared to the control groups (G1 and G2).

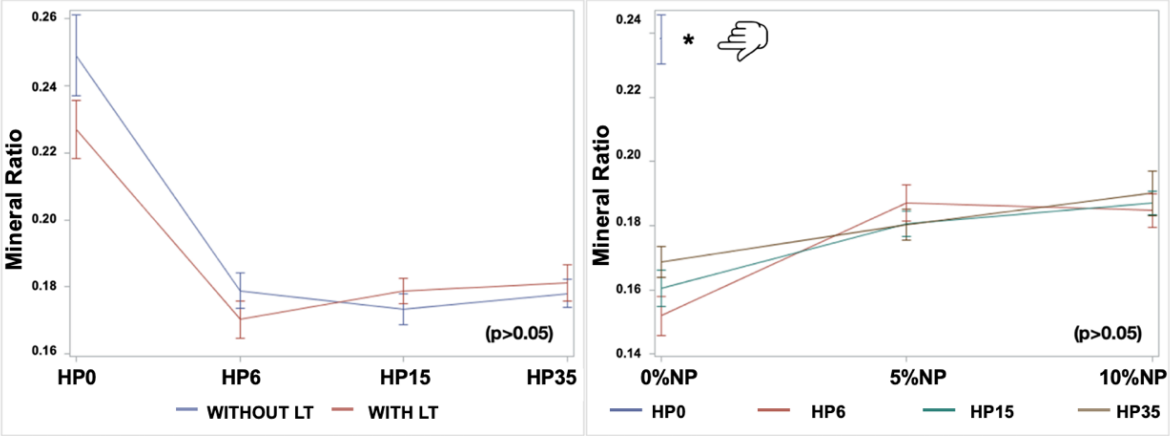

Figure 6. Carbonate:phosphate mineral ratio marginal means and standard errors within each level of HP (0%, 6%, 15%, 35%), NP (0%, 5% and 10%), and LT (with or without) according to the two-way interactions HP*LT and NP*HP, respectively. The finger icon points to the control group (HP0), and the asterisk represents the difference among the control and the other groups, taking into consideration a 0.05 level of significance

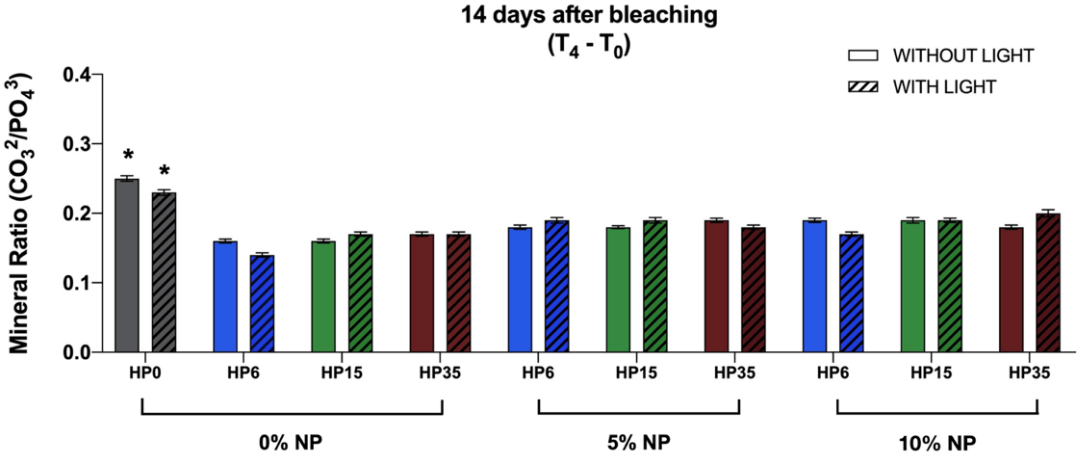

Figure 7. Mean and standard error values of the mineral ratio acquired after 14 days from bleaching (T_4) from the integrated areas of CO_3^2 υ2 to PO_4^3 υ1, υ2 contours. The asterisks represent the difference among the control groups and the other ones, taking into consideration a 0.05 level of significance

Figure 8 (A-T) illustrates the results from the ATR-FTIR analysis of the mineral content of enamel before (baseline [T_0], black curves) and after bleaching (14 days after

[T_4], red curves). Figure 8A indicates (in terms of normalized absorbance values) that specimens pertaining to G1 (negative control) displayed spectra characterized by absorbance values that were slightly higher at T_4 (wavenumbers from 800 cm^{-1} to 1,150 cm^{-1} and from 1,350 cm^{-1} to 1,550 cm^{-1}) and by a larger spectral bandwidth at absorbance values between 0.0 and 0.5. In combination, these results indicate that the mineral content of enamel was not altered by storing specimens in artificial saliva for the duration of the experiment. Similar behavior was observed in Figure 8B, which demonstrates that the utilization of violet light irradiation (in the conditions tested), also did not promote any changes to the chemical composition of treated enamel.

Figures 8 (C, I and O; HP [6%, 15% and 35%]), (F, L, and R; HP [6%, 15% and 35%] + LT), (D, J and P; HP [6%, 15% and 35%] + NP [5%]), (G, M and S; HP [6%, 15% and 35%] + NP [5%] + LT), (E, K and O; HP [6%, 15% and 35%] + NP [10%]) and (H, N and T; [6%, 15% and 35%] + NP [10%] + LT) illustrate the results for specimens that were subjected to experimental bleaching protocols. It is possible to observe that specimens treated with HP (either 6%, 15%, and 35%) with or without LT (Figures 8C, 8F, 8I, 8L, 8O, and 8R), displayed spectra at T_4 that was characterized by lower absorbance values for the CO_3^2 $\upsilon 2$ (886 cm^{-1}) and PO_4^3 $\upsilon 1$ (996 cm^{-1}) peaks. In addition, it was possible to observe that the combination of HP and LT shifted the spectra to the right (wavenumbers between 800 cm^{-1} and 1,150 cm^{-1}). Such behavior was more drastic for specimens treated with either 6% or 15% HP and LT (Figures 8F and 8L). This behavior was not observed on specimens treated with bleaching gels containing NP independent of light irradiation (with or without) or nanoparticles' concentrations (either 5% or 10%). In these instances, spectra were observed to display shapes and absorbance values that were similar to those of the control group (no treatment, stored in artificial saliva) at T_4.

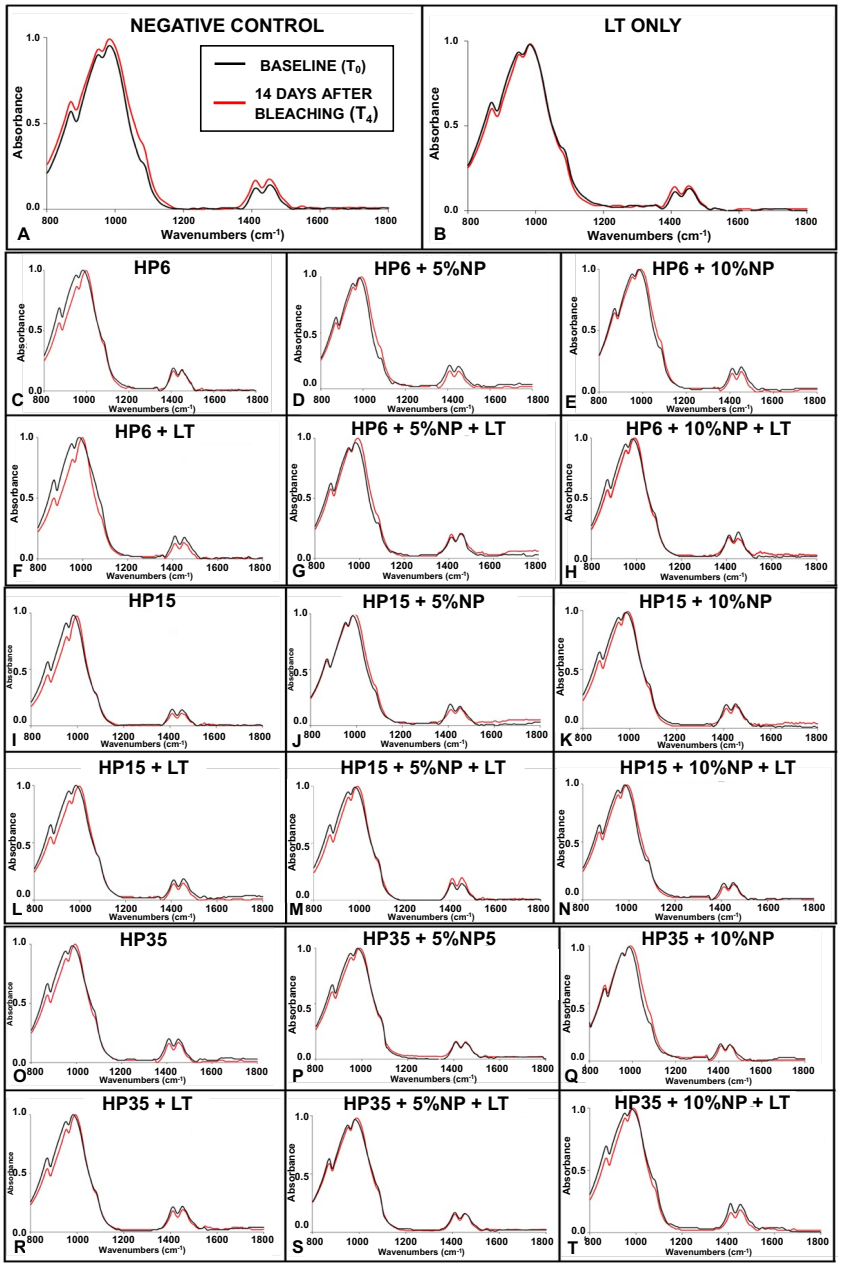

Figure 8. ATR-FTIR spectra of specimens before (black curves, [T_0]) and 14 days after (red curves, [T_4]) being treated with experimental bleaching gels containing HP (6%, 15% and 35%) with or without NF_TiO_2 and with or without visible light irradiation (LT)

Topography Assessment

Illustrative results from the topographical assessment performed with AFM are shown in Figure 9 (A – NN) where it is possible to observe that R_a and R_q values varied from 1.5 nm (HP35+5%NP at T_4) to 19.6 nm (HP6+10%NP) and 2.1 nm (HP35+5%NP) to 25.2 nm (HP6+10%NP), respectively. It is possible to observe through the images that, in most cases, the topography of the surfaces was maintained between T_0 and T_4. Overall,

the surfaces were smooth and few of them illustrated the distribution and direction of enamel prisms.

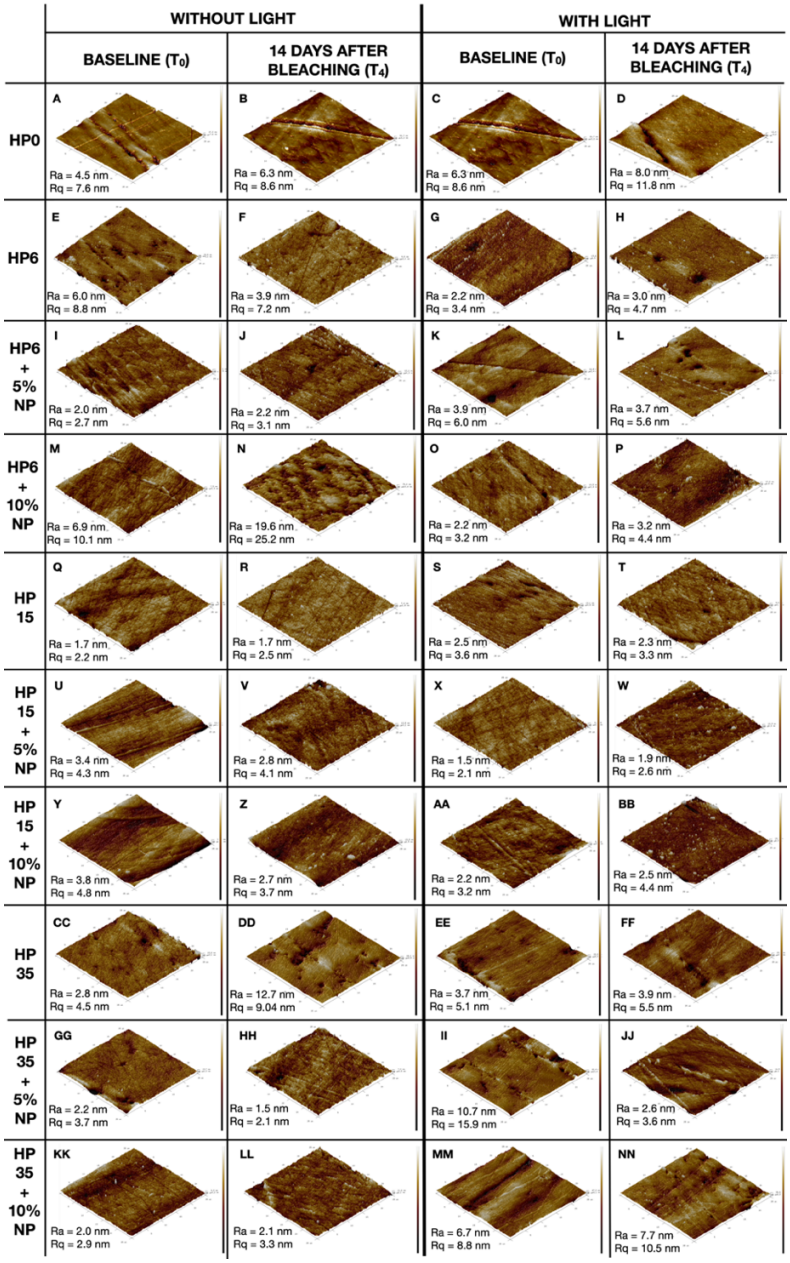

Figure 9. Images acquired using atomic force microscopy showing illustrative areas of the enamel before (T_0) and after 14 days (T_4) from bleaching with HP6, HP15 and HP35, with corresponding %NF_TiO_2 and presence or absence of LT. Control groups are represented by HP0

Metabolic status of non-disrupted biofilms

The general linear model evaluation detected significance for both isolated factors group and time ($p < 0.0001$), but no interaction was reported among them (group*time, p

= 1.000). Figure 10 illustrates the results, in terms of relative luminescence units (RLU) [mean and standard deviation values 6 minutes after the addition of D-Luciferin], of the metabolic status of non-disrupted *S. mutans* biofilms grown for 24 hours on the surface of the specimens that were previously bleached. It is possible to observe that, except for G9 (HP6+5%NP), biofilms grown on specimens treated with experimental bleaching protocols modulated by gels containing HP (6%, 15% and 35%) with (5% and 10%) or without NP and LT, displayed metabolic statuses that were either comparable to or higher than those observed on G1 (negative control).

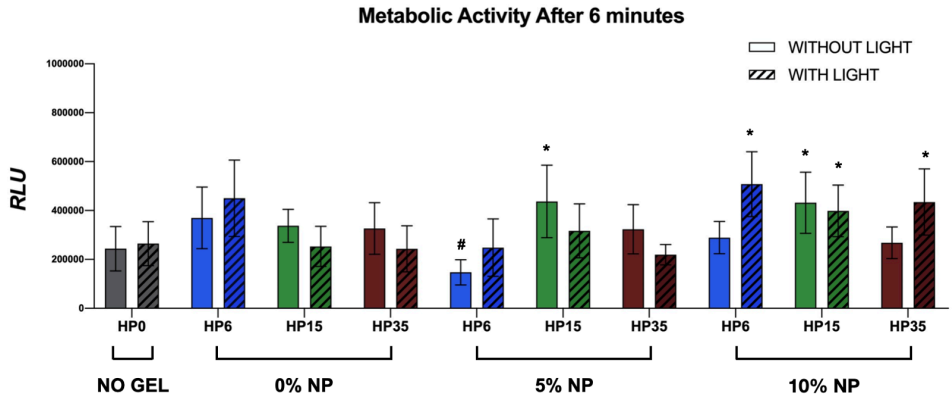

Figure 10. Mean and standard deviations of RLU values 6 min after the addition of D-Luciferin substrate to 24-hour *Streptococcus Mutans* (JM10) biofilms. S. mutans biofilms were grown on the enamel of individual specimens 14 days after the last bleaching session (T_4). The hashtag sign denotes groups with RLU values that were statistically lower than those from the control group (no treatment - G1), while asterisks represent groups with RLU values that were statistically higher than those from G1

Confocal microscopy

The results from the concurrent staining and confocal laser scanning microscopy analysis are show in Figure 11 (A – R) as 3-D reconstructions of biofilms, where it is possible to observe green (nucleic acids), red (proteins) and blue (EPS) fluorescence channels, the impact of experimental bleaching treatments on components of biofilms and their 3-dimensional distributions. It was possible to detect in figure 11A, that biofilms expressed mostly green fluorescence when grown against the surfaces of specimens that were not treated with experimental bleaching gels (G1 – negative control). It was possible to observe that biofilms grown on groups that were not light-irradiated surfaces were not only more porous, but also displayed fluorescence signals that were mostly red and blue, which indicates that biofilms were expressing proteins and EPS more intensely. A clear trend, in terms of fluorescence signals (either green, red, or blue), could not be observed

for specimens treated with experimental bleaching gels containing HP (6%, 15% or 35%) with (5% and 10%) or without NP and LT.

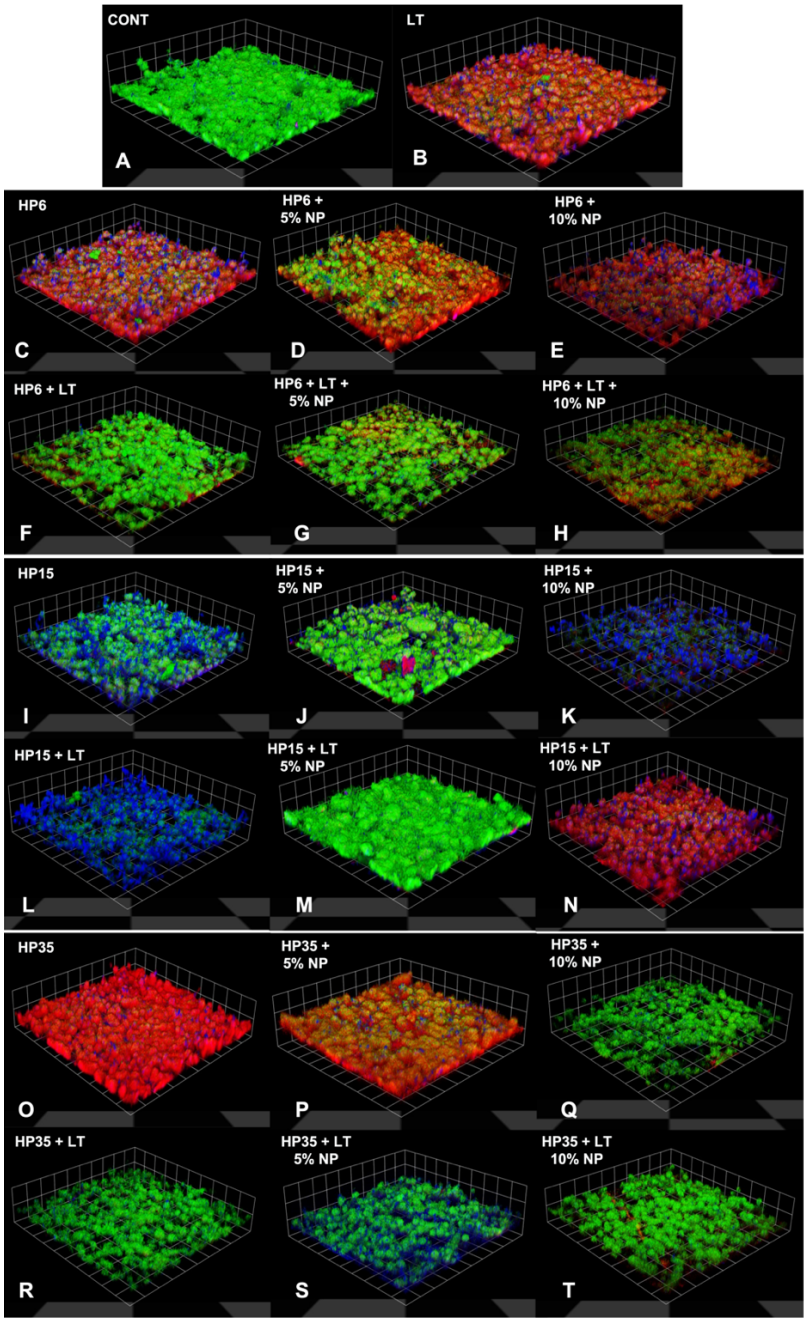

Figure 11. 3-D reconstructions of the biofilms with concurrent staining observed using confocal laser scanning microscopy 14 days after (T_4) bleaching with 6%, 15% and 35% HP with or without incorporation of 5% and 10% NP. It is possible to detect the distribution of the nucleic acid (green fluorescence), proteins (red fluorescence), and EPS (blue fluorescence) components of biofilms. Biofilms grown on surfaces bleached without LT were more porous, exhibiting mostly red and blue fluorescence signals. A clear trend, in terms of fluorescence signals (either green, red, or blue), could not be observed for specimens treated with experimental bleaching gels containing HP (6%, 15% or 35%) with (5% and 10%) or without NP and LT

DISCUSSION

Even though the efficacy of IPB [31] has been previously demonstrated by numerous research groups, post-treatment DH continues to be the most frequently reported adverse effect [15-17]. In fact, a previous study [36] investigating the correlation between bleaching efficacy and risk/intensity of post-treatment DH has indicated, based on a multi-regression and logistic analysis, that the risk for the occurrence of DH was 120% more likely to precipitate from IPB than from at-home bleaching techniques [36]. In addition, the intensity of painful symptoms was reported to be at least four times stronger for patients treated with IPB than those subjected to at-home treatments.

According to previous studies, the intensity (low, mild, and severe) and duration (short-term or long-term) of DH precipitates directly from peroxide concentrations and exposure times used [17]. Therefore, the behavior reported [36] is expected because at-home bleaching gels are three-to-six times less concentrated than those used in IPB [1-3], were demonstrated to be less cytotoxic and to penetrate less into the tooth structure [6, 17], thereby diminishing potential risks associated with the vitality of pulpal tissues. Despite these promising results, at-home techniques require long exposure times, and result in bleaching outcomes that are only similar to those achieved with IPB [37]. This critical scenario underscores the need for the development of techniques and products that are capable of resolving dental discolorations in a short period of time and without causing DH or negatively impacting the properties of teeth (surface, mechanical and biological).

The present research represents an effort to overcome limitations cited by developing experimental bleaching gels containing low concentrations of HP, and third-generation titanium dioxide nanoparticles co-doped with nitrogen and fluorine, that have been demonstrated to have non-leaching antibacterial properties [25, 27]. Results reported in the present study have demonstrated that the incorporation of NP (5% and 10%, w/v) into experimental bleaching gels containing low concentrations of HP (6% and 15%) rendered esthetic outcomes (in terms of ΔE_{00} and ΔWI_D) that were similar to those attained with high-concentrated bleaching gels (HP35), thereby suggesting that the nanotechnology proposed has the potential to resolve mild-to-severe dental discolorations in short periods of time (3 sessions, 30 min/session) and with lower amount of hydrogen

peroxide. Therefore, the first null hypothesis that NP incorporation would not significantly influence the efficacy of experimental bleaching gels was rejected. The utilization of violet light irradiation (LT; 405 nm ± 15 nm) was shown to improve the efficacy of experimental bleaching gels containing varying concentrations of HP (6%, 15% and 35%) with or without NP (5% and 10%), as denoted by the statistical outcomes and the mean values of ΔE_{00} and ΔWI_D that were higher than those from experimental bleaching gels (with or without NP) in dark conditions, which demonstrates that LT is still fundamentally important to achieving good esthetic outcomes.

Tano et al. [38] while investigating the effects of visible light irradiation (405 nm) emitted from a laser source on the efficacy of HP35 + TiO_2 (0.1% wt/wt) against a methylene blue solution (MB; 1.0 g, 100 ppm in 7.0 g of water), have demonstrated that even small concentrations of HP and TiO_2 can be efficiently used to bleach organic dyes when in the presence of visible light, thereby further supporting the necessity of using light irradiation and the present study's rationale for the selection of concentrations investigated (HP and NP).

Despite these promising in vitro results, findings from randomized clinical trials (RCTs) investigating the efficacy of HP6 with either TiO_2 [39] or N_TiO_2 [22, 40] were less encouraging, and have shown that experimental bleaching gels tested [22, 39, 40], produced less DH but were much less effective (in terms of bleaching outcomes) when compared to commercially-available products (35% HP). These findings could have precipitated from the combination between the wavelength selected (450 nm ± 15 nm; 2.76 μeV) [22, 40] and bleaching gels containing nanoparticles fabricated by calcination strategies, that behave as semiconductors and cannot generate ROS. In combination, factors cited result in sub-optimal bleaching reactions and poor esthetic outcomes.

The experimental design of the present study was based on the utilization of shorter wavelengths (405 nm ± 15 nm) with higher photon energies (3.06 μeV), and nanoparticles (NF_TiO_2; 6-15 nm) that were synthesized using robust and green solvothermal reactions as previously reported by our group [25, 27, 28]. Nanoparticles reported herein were shown to have well-defined pore-size distribution, are electron deficient, are capable of producing substantial amounts of ROS [26], and result into experimental materials that are more efficient and less aggressive to the tooth structure. A previous study [25]

demonstrated that single-doped nanoparticles (N_TIO_2), fabricated using similar synthetic routes, were capable of absorbing twice as much light (between 200 nm – 800 nm) as compared to commercially available nano-TiO_2 (P25, Degussa) [25]. Since LT has been demonstrated by the present study to be fundamentally important for the success of IPB, and taking into consideration that nanoparticles reported have the ability to intensely absorb visible wavelengths, it can be hypothesized that experimental protocols investigated could result in good esthetic outcomes.

This hypothesis has been corroborated by findings reported in a recent randomized, controlled and double blind clinical trial [41] that investigated the clinical performance (in terms of immediate ΔE_{ab}) of 6% HP with N_TiO_2 when activated by two distinct wavelengths (405 $\pm$ 15 nm and 450 $\pm$ 15 nm). The results of that study [41] show that the clinical color change of experimental bleaching gels was less pronounced when using blue irradiation (450 $\pm$ 15 nm). The authors have also reported that bleaching protocols modulated by violet radiation displayed bleaching efficacies that were comparable to the control group (35% of HP) but were associated with lower levels of DH. Even though the trends observed [41] validate the results of the present study, it is important to underscore that our experimental design was based on the utilization of the CIEDE2000 formula (ΔE_{00}) [32] and the whiteness index (ΔWI_D), which are considered more relevant for dentistry and dental bleaching investigations.

According to Paravina et al. [42] the mean ΔE_{00} values reported for G10 (HP6+NP5+LT) are considered excellent and indicate that experimental protocols modulated by LT and 5% of NP resulted in bleaching efficacies that were comparable to those from HP15 (G6, G11, G12) and from HP35 (G8, G14, G19, G20). These findings could have precipitated from the spontaneous dissociation that hydrogen peroxide undergoes when in the presence of metaloxides such as NF_TiO_2 and suggest that experimental bleaching gels containing low concentrations of NP and HP may result in materials with promising bleaching performance. The higher mean values of ΔE_{00} and ΔWI_D detected in G11 (HP15+NP5) serve as additional evidence of such spontaneous dissociation process. This was an expected behavior and has been corroborated by previous research from our group that demonstrated that photocatalysts of similar

compositions displayed promising antibacterial properties against *Streptococcus mutans* even in the absence of light irradiation [27].

With regard to the temporal evolution of pH, it was possible to observe that the incorporation of NP (5% and 10%) into experimental bleaching gels containing HP (6%, 15%, and 35%) did not adversely impact the values observed. For experimental gels containing 6% HP, the NP incorporation (either 5% or 10%) resulted in pH values that were higher when compared to gels without NP (with or without LT). Gels containing either 15% or 35% of HP, displayed pH values that fluctuated a bit more, but overall, the incorporation of NP seemed to have a stabilizing effect that prevented the acidification of experimental materials during the investigated period of time (30 min). Our findings contradict the results published by Pretel et al. [43], because, even though commercial gels modified by the incorporation of N_TiO_2 (produced by calcination strategies) displayed initial pH values that were high, materials investigated [43] displayed pH values that were significantly lower after 30 minutes of observation. On the other hand, Monteiro et al. [44] have shown that the incorporation of 1% TiO_2 nanotubes did not adversely impact the pH values of gels containing either 10% carbamide peroxide (pH $\cong$ 6.5) or 40% HP (pH $\cong$ 7.0). These differences in pH values reported in the literature and by the present study (initial and after 30 minutes) can be explained by the intrinsic differences in materials' compositions including the type of polymeric matrix and stabilizing agents.

A recent study [43] investigating temporal variations of pH and electric potential (EP) of three commercially available bleaching gels has demonstrated that there is a strong and positive correlation between pH and EP values, where EP inversely varied with the evolution of pH. Gentil de Moor et al. [45] have indicated that ROS such as oxygen, hydroperoxyl, sodium hypochlorite, hydrogen peroxide, ozone, and hydroxyl have redox potentials of +1.229 V, +1.510 V, +1.630 V, +1.780 V, +2.075 and +2.800 V, respectively, and therefore, bleaching gels proposed in the present study are hypothesized to preferentially generate oxygen and hydroperoxyl and to prevent the etching of treated enamel.

In our study, a significant decrease in the mineral ratio was detected for all bleached groups in comparison to the control ones, independent of the NP incorporation. Therefore, the second null hypothesis that the NPs would not negatively influence the

chemical content of enamel bleached with the experimental gels was rejected. However, the results of the present study suggest a trend of increasing the mineral ratio of enamel bleached with gels containing NP (5% and 10%), which should be further confirmed with additional mechanical and surface testing. Xu et al. [46] already demonstrated the presence of a strong and inverse relationship between the carbonate:phosphate ratio and surface properties of enamel (elastic modulus and hardness). Even though the location of the ATR-FTIR evaluation over time (T_0 and T_4) was standardized, the specimens were stored in saliva for 14 days after bleaching as an additional attempt to mimic as much as possible the clinical condition. [9, 10] As it has already been demonstrated, the presence of saliva may either uphold or recover the levels of mineralization of enamel after tooth bleaching [47].

Another valuable information provided by the ATR-FTIR assessment was the spectra of enamel, demonstrating that experimental bleaching gels containing HP (6%, 15% and 35%) but without NP (which are more acidic) have negatively impacted the chemical make-up of treated enamel independently of LT, as denoted by a right-shift of the spectrum between wavenumbers between 800 cm^{-1} and 1,150 cm^{-1} and lower absorbance values for CO_3^2 $\upsilon 2$ (886 cm^{-1}) and PO_4^3 $\upsilon 1$ (996 cm^{-1}). As demonstrated in Figure 5, this behavior was overall not observed on specimens treated with experimental bleaching gels containing NP (either 5% or 10%). In these instances, spectra were observed to have shapes and absorbance values that were similar to those of the control group (no treatment). Although some authors have reported distortions in the peaks mentioned independently of pH values, [46, 48] Sun et al. [7] have also demonstrated that acidic bleaching agents (30% HP, pH $\cong$ 3.6) not only decreased CO_3^2 $\upsilon 2$ (886 cm^{-1}) and PO_4^3 $\upsilon 2$ (1,410 – 1,460 cm^{-1}) absorbance values but have also right-shifted the spectra (between 800 cm^{-1} and 1,150 cm^{-1}) of treated enamel, thereby further corroborating the findings of the present study. In their study [7], the decrease in the mineral content (CO_3^2/PO_4^3) was followed by significant reductions in enamel's surface microhardness. This concerning trend was not detected when authors have used experimental bleaching agents with neutral pH [7].

It is important to highlight that the evaluation of the carbonate (CO_3^2) content provides valuable information to the field because CO_3^2 represents 2.5 weight % of

enamel's composition, behaves as a substitute anion for phosphate or hydroxyl groups in hydroxyapatite[45] (HAp [$Ca_{10}(PO_4)_6(OH)_2$]) and is not stoichiometrically distributed in dental enamel[46]. In addition, the carbonate's position (at hydroxyapatite's lattice or surface) not only modifies its shape and size, but more importantly, increases the solubility of carbonated apatites [49, 50]. This physico-chemical property of hydroxyapatite could be used to explain the reason why experimental bleaching gels without nanoparticles significantly decreased the mineral ratio of treated enamel. We hypothesize that the trend of increasing mineral ratio observed when specimens were treated with gels containing NP (5% and 10%) could be explained by the presence of fluorine ions in the crystal lattice of NF_TiO_2 that could potentially alter the composition of hydroxyapatite (into fluorapatite) through an ion-exchange mechanism. Even though the mechanism is not energetically favorable, the formation of fluorapatite could be responsible for the maintenance of the chemical make-up observed once fluorapatite displays solubility levels that are lower to those of carbonated apatites when exposed to acidic pH values [49, 50].

Even though large variations in R_a and R_q values could be correlated to intense damage to enamel, the vast majority of our results have indicated that treatments conducted with or without LT, and modulated by experimental gels containing (6%, 15% and 35%) with (5% and 10%) or without NP, were not able to adversely impact the surface topography of treated enamel at T_4, and therefore, results from the group treated with 6HP+10%NP were considered to not follow the overall trend observed. Additional analyses based on optical profilometry will include a quantitative evaluation of enamel's surface roughness. Contrarily to these findings, others [7, 12] have shown that enamel surface properties were negatively impacted by bleaching protocols modulated by high-concentrated bleaching gels (either 30% or 40% HP), independently of their pH values (either acidic or neutral), using similar AFM techniques. Therefore, it could be assumed that compositions tested have the potential to resolve mild-to-severe dental discolorations without negatively impacting the chemical arrangement or surface properties of treated enamel.

These findings are also important from the oral microbiology standpoint because it is well-known that surfaces (either biotic or abiotic) with high mean R_a values accumulate more biofilms [51] due to increased surface area and surface energy, and may alter the

ecology of biofilms from a state of health into a disease-associated state. The real-time and high throughput bioluminescence assay performed in the present study had the objective to determine (i) if enamel surfaces treated would display a latent antibacterial behavior and (ii) if surfaces treated would support more biofilm growth. As demonstrated by the results shown in Figure 10, experimental bleaching treatments modulated by HP (6%, 15% and 35%) and NP (5% and 10%) were not capable of rendering antibacterial effects to treated surfaces against non-disrupted biofilms of *Streptococcus mutans* independently of LT, as denoted by RLU values that were either similar or higher to those from the control group (no treatment). Therefore, the last null hypothesis that the incorporation of NP would not avoid growth of biofilm after bleaching was accepted. In the past, Ittatiruti et al. [51] demonstrated, using the viable colony counting assay (CFU/mL), that dental bleaching procedures modulated by bleaching gels containing either 25% or 35% of HP, did not promote higher accumulations of *S. Mutans*, but increased the *S. Sanguinis* biofilm formation.

The only exception to the trend observed in our work was on specimens from G9 (HP6+NP5) that displayed biofilms with RLU values that were slightly lower than those from the control group (no treatment). These findings suggest that enamel surfaces treated with HP6+5%NP could potentially become antibacterial by the utilization of the nanotechnology proposed, but additional studies are made necessary to confirm these findings and elucidate potential mechanisms of action associated. The qualitative results from the confocal microscopy analysis (Figure 11) have indicated that enamel surfaces treated with experimental bleaching protocols modulated by HP (6%, 15% and 35%) and NP (5% and 10%) with or without LT, promoted the growth of biofilms displaying different 3-dimmensional distributions of nucleic acids, proteins, and EPS, thereby suggesting that bleached enamel may indeed impact the accumulation and growth of oral biofilms. However, these results should be interpreted with caution because qualitative data reported cannot be considered representative due to the small number of specimens analyzed.

Subsequent studies from our group will investigate the mechanisms of action by which nanoparticles proposed (i) improve the efficacy of experimental bleaching gels, (ii) maintain the mineral content of treated enamel and (iii) modulate the 3-dimensional

distribution of biofilms' components. Our group is also planning the execution of a controlled, randomized and double-blind clinical trial to determine the clinical efficacy of experimental bleaching gels herein proposed.

CONCLUSIONS

The present study has successfully demonstrated the synthesis of experimental bleaching gels using hydrophilic polymers and functionalized nanoparticles. The nanotechnology reported was demonstrated to significantly improve the bleaching efficacy of experimental materials independent of hydrogen peroxide or light irradiation, and did not adversely impact surface properties or chemical make-up of treated enamel. The results of the present study have shown that experimental materials were not capable of rendering antibacterial effects to treated enamel but were observed to alter the 3-dimensional distribution of components within *S. mutans* biofilms. Subsequent studies should not only investigate the mechanisms of action by which the nanotechnology proposed improves bleaching reactions but also, demonstrate the clinical efficacy of the materials proposed.

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2.4 Artigo 4. Effects of experimental in-office bleaching gels incorporated with co-doped titanium dioxide nanoparticles on dental enamel physical properties

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ABSTRACT

Objective: To evaluate the physical properties of bovine dental enamel submitted to in-office experimental bleaching gels with hydrogen peroxide (HP) and incorporated with titanium dioxide nanoparticles (NP) co-doped with nitrogen and fluorine and irradiated with violet LED light (LT).

Methods: Enamel-dentin disks were randomly allocated (T_0) into groups, according to the concentrations of HP (HP6, HP15, or HP35) and NP (no NP, 5NP, or 10NP), and irradiated or not with LT. A negative control (NC) group, without treatment, was set. After three bleaching sessions (T_1, T_2 and T_3), specimens were stored in artificial saliva for 14 days (T_4). Enamel Knoop microhardness number (KHN) was obtained from T_0 to T_4, and surface roughness (*Ra*) at T_0 and T_4. At T_4, the disks were cut into halves to assess the cross-sectional microhardness (ΔS), energy dispersive spectroscopy (EDS), scanning electron (SEM) and polarized light (MLP) microscopies.

Results: Surface KHN was significantly influenced by NP over time, independently of LT irradiation. At T_3 and T_4, HP gels incorporated with either 5NP or 10NP exhibited no differences in KHN in comparison to NC and baseline values, which was not observed under the absence of NP. The addition of NP did not statistically interfere in the ΔS and *Ra*. MLP images exhibited enamel surface/subsurface darkening areas suggestive with demineralizing regions. SEM demonstrate some intraprismatic affection in the groups without NP. EDS reported a higher calcium:phosphorous ratio for enamel bleached with 10NP gels.

Conclusion: The incorporation of NP into experimental gels maintained the enamel surface microhardness levels and seemed to controlled enamel surface morphology, upholding the mineral content. None of the proposed experimental bleaching protocols

have negatively influenced the enamel surface roughness and the cross-sectional microhardness.

Clinical Significance: The application of in-office bleaching gels incorporated with co-doped titanium dioxide nanoparticles onto the enamel surface would not harm its physical properties.

Key words: Tooth Bleaching, Hydrogen Peroxide, Light Irradiation, Nanoparticles, Enamel Surface.

INTRODUCTION

In-office tooth bleaching holds the advantage of immediately resolving tooth discoloration by using high-concentrated hydrogen peroxide (HP) gels [1]. Nonetheless, there are several reports in the literature showing that application of these bleaching gels onto the enamel surface could cause adverse effects to the physical properties of enamel surface and its underlying subsurface. For instance, 35 to 40% HP decreased the enamel surface and cross-sectional microhardness [2-4], increased the surface roughness [5, 6], and negatively influenced the enamel surface morphology and mineral content [7, 8].

Such conditions could be a result from the oxi-reductive action of hydrogen peroxide, which penetrates into dentin through the interprismatic spaces of enamel [9]. The pH of the gels is also a feasible cause of the enamel surface alterations, possibly due to an ion-exchange mechanism. Efforts have been made to overcome these drawbacks, such as the incorporation of calcium and fluoride ions [10, 11] into experimental and commercial bleaching gels and the increase in the gel's pH [12]. Indeed, some studies have attested that such ions incorporation was capable of minimizing the microhardness loss due to tooth bleaching [13, 14].

The reduction of HP concentration also could be considered an interest approach because it could attenuate the oxidative reactions occurring on the enamel surface as well as directly influence in the amount of reactive oxygen species (ROS) or unreacted HP reaching the pulp chamber and, consequently, reducing the risk for tooth sensitivity [15, 16]. However, this approach should be accompanied by alternatives to maintain the optimal immediate esthetic outcomes from high-concentrated bleaching. In this direction, the incorporation of catalysts i.e., titanium dioxide, ferrous sulfate, catalase, manganese oxide, into the gels could accelerate or increase the generation of ROS that would interact with the so-called chromophores molecules in dentin and turn teeth whiter at the same level reported for high concentrations of HP [17-20].

Recently, the *in vitro* incorporation of titanium dioxide nanoparticles co-doped with nitrogen and fluorine into experimental in-office bleaching gel allowed up to five times reduction in the HP concentration (6% and 15%) with the maintenance of the optimal immediate color and whiteness index changes levels [21]. However, this was only possible under the light irradiation with a violet LED light. It was previously shown that the doping

of this titanium dioxide nanoparticle rendered optical absorption spectrum in the violet light range (λ = 390-420 nm) twice as much a commercially available nanoparticle (P25, Degussa) [22]. Briefly, when the nanoparticle interacts with an appropriate light wavelength, the electrons from the valence band (fundamental state) are promoted into the conduction band (excited state). Then, the electron vacancy, in the valence band, is positively charged and will recombine with the conduction's free electrons, releasing heat or light [23]. In case these electrons do not recombine, generation of positive and negative electrons may occur and participate in various oxi-reduction reactions in the surface of the nanoparticle, including the generation of longer-living reactive oxygen species [24].

Interestingly, the low-concentrated experimental gels incorporated with the co-doped nanoparticles also preserved the shape and absorbance values of carbonate and phosphate peaks of dental enamel under a FT-IR evaluation [21], presenting an upregulation trend of the enamel mineral ratio, contrarily to the gels without the nanoparticles. However, a detailed assessment of the enamel physical properties is paramount to determine the real effect of these nanoparticles-enriched gels on the enamel surface. Therefore, the objective of this study was to evaluate the effects of light-irradiated experimental in-office bleaching gels incorporated with titanium dioxide nanoparticles (NP) co-doped with nitrogen and fluorine on enamel surface microhardness, roughness, morphology, calcium/phosphorous content, and cross-sectional microhardness. The null hypotheses tested were that the incorporation of the NP into the experimental gels would not affect the enamel (i) microhardness nor the (ii) surface roughness.

MATERIAL AND METHODS

Experimental Design

The specimens (n = 190) were prepared and randomly allocated into groups (n = 10/group), according to the study factors:

- *Hydrogen Peroxide Concentration (HP):*
  - HP6: experimental bleaching gel with hydrogen peroxide at 6%
  - HP15: experimental bleaching gel with hydrogen peroxide at 15%
  - HP35: experimental bleaching gel with hydrogen peroxide at 35%
- *Nanoparticles Concentration (NP):*
  - Without NP
  - 5NP: experimental bleaching gel incorporated with 5% of NP
  - 10NP: experimental bleaching gel with 10% of NP
- *Light Irradiation (LT):*
  - Dark conditions
  - Light irradiation (LT)
- *Time (T) – depending on the variable response:*
  - Baseline *(T_0)*
  - After the first bleaching session *(T_1)*
  - After the second bleaching session *(T_2)*
  - After the third and last bleaching session *(T_3)*
  - After 14 days maintained in artificial saliva *(T_4)*

A group was maintained only in artificial saliva for the duration of the study (negative control). The specimens were submitted to enamel surface (from T_0 to T_4) and cross-sectional (T_4) microhardness analyses, surface roughness evaluation (T_0 and T_4), polarized light and scanning electron microscopies (T_4), and energy-dispersive X-ray spectroscopy (T_4).

Specimen's Preparation

Two-hundred and fifty bovine incisor teeth were collected, cleaned, and stored at 4^0C for no longer than 30 days. These teeth were then positioned in a water-cooled holder

coupled to a to a bench drill (Pratika FSB16P, Schultz, Joinville, SC, Brazil). A diamond bur for glass (⌀8mm, Di Martino Brocas Diamantadas Ltda, Campinas, SP, Brazil) was used to obtain enamel-dentin disks (diameter = 5.6 mm) from the middle third of the incisor's buccal surface. The dentin end of the disks was abraded with 600-grit sandpaper (Norton Saint-Gobain, Guarulhos, SP, Brazil) using a rotary polisher (Arotec, São Paulo, SP, Brazil), and the outer enamel surface was abraded with 400- and 1200-grit sandpapers (Norton Saint-Gobain) and polished using polishing cloths (3M Brazil, Sumaré, SP, Brazil) with diamond suspensions (1 μm, 0.50 μm and 0.25 μm, Erios, São Paulo, SP, Brazil). The final diameter of the disks, checked with a digital caliper (Cisel, São Paulo, SP, Brazil), was equal to 2.5 mm (enamel = 1mm, dentin = 1.5 mm). Immediately after the specimens' preparation and before any bleaching procedures or immersion in artificial saliva (T_0), the specimens were tested to obtain the initial Knoop surface microhardness number (KHN). Taking into consideration that the entire set of specimens presented a 350.0 ± 41.0 KHN mean, those specimens with KHN 10% higher or lower than the mean were excluded from the research.

Nanoparticles' Synthesis and Experimental Gel's Preparation

The synthesis of NP was previously described [21, 22]. The reagents - 1.7 g of Ti $(OBu)_4$ (97%, Sigma-Aldrich, St. Louis, MO, USA), 4.6 g C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, USA), 6.8 g $C_{18}H_{35}NH_2$ (Sigma-Aldrich, 70%), 7.1 g $C_{18}H_{34}O_2$ (Sigma-Aldrich, 90%) and 5% of NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar, Haverhill, MA, USA) - were mixed with an ethanol-water solution into a high-pressure reaction vessel (Borosilicate Glass-lined; Paar Series 4593, Bench Top Reactor System, Moline, Il, USA). This vessel reacted at 180 °C (15 psi) and stirred at 280 rpm for 24h. The solution was then dispensed into a falcon tube containing ethanol (200-proof, Decon Labs, King of Prussia, PA, USA) and centrifuged for 15 min at 8,000 rpm three times. The preparation of the experimental gels was also previously reported in detail [21]. A commercial hydrophilic polymer (Carbomer 940 NF, Spectrum, Gardena, CA) was diluted into ultrapure water by means of a planetary and orbital stand-alone mixer (Speed Mixer, DAC 400.1 FVZ, FlackTek Inc, Laudrum, SC, USA). The pH of this experimental gel was around 6.0, before being mixed with HP solutions. Aliquots of the co-doped nanoparticles

(1 and 2 mL of NP, ~ 40 mg/mL) suspended in ethanol were poured into separated falcon tubes and centrifuged at 8,000 rpm during 5 min. The ethanol was removed from the tube and the NPs were incorporated into 20 g of the experimental gels, which were mixed at 2,450 rpm for 20 s (Speed Mixer, DAC Iso.1 FVZ, FlackTek Inc, Laudrum, SC, USA). As a result, the resulting gels contained 5 and 10% of NP (v/w), respectively.

Groups Division and Bleaching Procedures

The 190 specimens selected were then randomly distributed into 19 groups (n = 10), according to the bleaching treatments with the experimental gels assigned to each one:

- **NC:** negative control, stored in artificial saliva throughout the study
- **HP6:** 6% hydrogen peroxide gel
- **HP6+LT:** 6% hydrogen peroxide gel, light-irradiated
- **HP15:** 15% hydrogen peroxide gel
- **HP15+LT:** 15% hydrogen peroxide gel, light-irradiated
- **HP35:** 35% hydrogen peroxide gel
- **HP35+LT:** 35% hydrogen peroxide gel, light-irradiated
- **HP6+5NP:** 6% hydrogen peroxide gel incorporated with 5% of NP
- **HP6+5NP+LT:** 6% hydrogen peroxide gel incorporated with 5% of NP and light-irradiated
- **HP15+5NP:** 15% hydrogen peroxide gel incorporated with 5% of NP
- **HP15+5NP+LT:** 15% hydrogen peroxide gel incorporated with 5% of NP and light-irradiated
- **HP35+5NP:** 35% hydrogen peroxide gel incorporated with 5% of NP
- **HP35+5NP+LT:** 35% hydrogen peroxide gel incorporated with 5% of NP and light-irradiated
- **HP6+10NP:** 6% hydrogen peroxide gel incorporated with 10% of NP
- **HP6+10NP+LT:** 6% hydrogen peroxide gel incorporated with 10% of NP and light-irradiated
- **HP15+10NP:** 15% hydrogen peroxide gel incorporated with 10% of NP

- **HP15+10NP+LT:** 15% hydrogen peroxide gel incorporated with 10% of NP and light-irradiated
- **HP35+10NP:** 35% hydrogen peroxide gel incorporated with 10% of NP
- **HP35+10NP+LT:** 35% hydrogen peroxide gel incorporated with 10% of NP and light-irradiated

The experimental gels were mixed with HP6, HP15 or HP35 solutions as previously reported [21]. The specimens were submitted to three 30-min bleaching sessions (T_1, T_2 and T_3) at 7-day intervals. The specimens were stored in artificial saliva [25] among the intervals and during 14 days after the last bleaching session. The LT groups were light-irradiated based on a protocol described elsewhere [26, 27].

Enamel Surface Microhardness

Enamel surface microhardness was determined using a Knoop microhardness device (Future Tech-FM-1e, Tokyo, Japan). Three indentations were performed in the central area of each specimen, 100-μm apart from each other under a static load of 50 g for 5 s [28]. The mean values were obtained (in Kgf/mm^2) at baseline (T_0) as described in the *Specimen Preparation* section, also 24 hours after each bleaching session (T_1, T_2 and T_3), and 14 days after the last bleaching session and storage of the specimens in artificial saliva (T_4).

Enamel Surface Roughness

Surface roughness (Ra, in μm) was determined by a surface tester (Mitutoyo Surftest SJ-410, Kawasaki, Japan) at T_0 and T_4. Three measurements were performed in each specimen by rotating the specimen 45º, with a cut-off set at 0.25 mm and speed of 0.2 mm/s [29].

Enamel Cross-Sectional Microhardness (CSMH)

After the final surface microhardness measurement at T_4, all the enamel-dentin disks were cross-sectionally cut into two halves. One half of each specimen was embedded in polystyrene resin (Future Tech-FM-1e, Tokyo, Japan), leaving the inner face exposed. The inner face of all specimens was polished with 400-, 600-, and 1200-grit sandpapers (Norton Saint-Gobain) during 5 min for each grit. CSMH analysis was

performed using the same microhardness tester aforementioned, using a Knoop indenter with a 25 g load for 5 s. The indentations were made 20, 30, 40, 50, 60, 80, 100, 120, 160 and 200 μm from the outer enamel surface [10]. The values were averaged, and the mean was expressed in KHN. ΔS was calculated for each specimen, representing the area of hardness loss calculated by numerical integration which uses a trapezoidal rule based on the difference between the area under the curve of sound enamel (kgf/mm^2 x μm) subtracted from area of the demineralized one [30].

Polarized Light Microscopy (PLM)

Some of the other halves was used for PLM and the inner surfaces of the samples (n=2/group) were polished to a thickness of 100 μm (± 0.1 μm) and mounted on glass plates with deionized water. The images were obtained under 200 × magnification [10] using a polarized light microscope (DM LSP, Leica Microsystems) to qualitatively evaluate the demineralization depth.

Scanning Electron Microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS)

Some of the other remaining halves (n = 2/group) were prepared to perform SEM and EDS analyses. The surface morphology was observed under SEM, and enamel calcium and phosphorus content were assessed by EDS. The specimens were dry-incubated (37^{0}C, 24 h), placed on an acrylic stub and carbon-coated in preparation for analysis in an EDS (Noran Instruments, Middleton, WI, USA) coupled to a SEM (JSM 5600 LV, JEOL, Tokyo, Japan). The SEM images were acquired at 500 x magnification. Five different locations of each specimen was selected in EDS to determine the calcium (Ca) and phosphorous (P) atomic weight (15 kVp, working distance = 20 mm, spot size = 55) [31], and the Ca/P ratio was then calculated.

Statistical Analyses

Data were submitted to exploratory analyses of normal distribution (Shapiro-Wilk) and homoscedasticity (Levene). The Ra values was transformed in Log_{10} to meet the assumptions for normal distribution and homoscedasticity ($p > 0.05$). Enamel surface microhardness (KHN) and roughness (Ra) data were submitted to four-way repeated

measures ANOVA. Since KHN exhibited statistical differences in some factors and interactions, pairwise comparisons were performed by post hoc Bonferroni test. The negative control group was tested against all the other experimental groups using the Dunnet test. ΔS data were analyzed by three-way ANOVA and Tukey test. All the statistical analyses were performed in the SPSS software (IBM, Chicago, Il, USA), at a significance level of 5%.

RESULTS

KHN

Table 1 depicts the mean and standard deviation values of KHN (kg/mm^2) over time. The NPs ($p < 0.001$) significantly influenced the KHN values. The factor time ($p < 0.001$), and the interactions time*HP ($p = 0.010$) and time*NPs ($p <0.001$) significantly influenced the results.

At baseline, no differences in KHN were noted among groups (T_0; $p > 0.05$). At T_1, a significant drop was detected in some groups in comparison to T_0. At T_1, HP6 and HP6+LT exhibited lower KHN than the negative control ($p < 0.05$), HP6 presented KHN lower than HP35, and HP6+LT lower than HP15+LT and HP35+LT ($p < 0.05$). After the second bleaching session (T_2), most NP-containing groups still exhibited comparable KHN to their baseline values and to NC, except HP6+5NP+LT and HP35+5NP+LT.

Immediately after bleaching (T_3), groups without NPs presented KHN statistically inferior to their corresponding baseline values and to NC, regardless of HP concentration and LT irradiation. Except for HP15+5NP (with or without LT), all groups without NPs promoted lower KHN than treatments performed with gels incorporated with NPs. Fourteen days after artificial saliva immersion (T_4), only the NP-containing groups maintained KHN compatible with baseline and NC values. HP6+LT kept KHN similar to baseline, but lower than NC.

Table 1. Means and standard deviation KHN values taking into consideration the enamel surface tested before bleaching (T_0), twenty-four hours after each one of the three bleaching sessions (T_1, T_2 and T_3) and 14 days of storage in artificial saliva (T_4)

NOLT		HP6						HP15						HP35					
		0NP		5NP		10NP		0NP		5NP		10NP		0NP		5NP		10NP	
	T_0	357.2(28.2) ^{Aa}	A	357.0(27.8) ^{Aa}	A	356.9(30.5) ^{Aa}	A	354.4(27.4) ^{Aa}	A	355.6(27.2) ^{Aa}	A	357.5(25.5) ^{Aa}	A	358.9(27.8) ^{Aa}	A	356.5(27.3) ^{Aa}	A	355.9(27.1) ^{ABa}	A
	T_1	286.5(39.9) ^{Cb*}	B	339.7(22.4) ^{Aa}	A	347.8(32.0) ^{Aa}	A	312.5(38.4) ^{BCb}	AB	351.6(36.2) ^{ABa}	A	361.5(34.5) ^{Aa}	A	321.4(46.3) ^{Ba}	A	358.9(37.6) ^{Ab}	A	351.0(31.9) ^{ABb}	A
	T_2	300.4(36.2) ^{Cb*}	A	351.7(32.7) ^{Aa}	A	361.6(29.3) ^{Aa}	A	301.2(17.1) ^{Cb*}	A	354.2(34.5) ^{ABa}	A	350.3(25.9) ^{Aa}	A	307.7(50.6) ^{Bb*}	A	336.8(42.7) ^{Aab}	A	369.3(33.5) ^{Ab}	A
	T_3	282.6(31.6) ^{Cb*}	B	348.2(26.0) ^{Aa}	A	351.8(20.7) ^{Aa}	A	315.8(28.5) ^{BCb*}	A	337.1(24.8) ^{Bab}	A	348.9(20.7) ^{Ab}	A	317.5(40.8) ^{Bb*}	A	358.7(22.2) ^{Aa}	A	344.6(25.4) ^{Ba}	A
	T_4	313.8(28.2) ^{Bb*}	A	358.2(17.4) ^{Aa}	A	350.2(17.4) ^{Aa}	A	328.5(27.1) ^{Bb*}	A	377.0(21.7) ^{Aa}	A	355.0(17.4) ^{Aa}	A	326.6(45.4) ^{Bb*}	A	351.1(37.9) ^{Aab}	A	360.3(26.1) ^{ABa}	A
		HP6						HP15						HP35					
		0NP		5NP		10NP		0NP		5NP		10NP		0NP		5NP		10NP	
	T_0	356.4(27.8) ^{Aa}	A	358.2(26.6) ^{Aa}	A	357.3(26.9) ^{Aa}	A	357.1(28.6) ^{Aa}	A	355.8(29.9) ^{Aa}	A	357.7(30.1) ^{Aa}	A	355.5(31.3) ^{Aa}	A	356.3(27.7) ^{Aa}	A	358.1(30.2) ^{Aa}	A
LT	T_1	280.5(43.5) ^{Bb*}	B	339.7(22.1) ^{ABa}	A	328.2(36.2) ^{Ba}	A	320.7(28.4) ^{BCb}	A	342.9(47.0) ^{Aab}	A	355.7(25.2) ^{Aa}	A	328.8(26.1) ^{Ba}	A	340.6(40.3) ^{ABa}	A	332.2(31.3) ^{Ba}	A
	T_2	302.6(39.7) ^{Bb*}	A	331.2(27.7) ^{Bab*}	A	344.9(25.9) ^{ABa}	A	308.1(15.7) ^{Cb*}	A	334.6(52.5) ^{Aab}	A	351.1(37.9) ^{Aa}	A	310.0(27.6) ^{Bb*}	A	323.4(33.9) ^{Bab*}	A	366.9(35.6) ^{Ab}	A
	T_3	292.0(16.6) ^{Bb*}	A	353.7(32.6) ^{ABa}	A	346.9(23.4) ^{ABa}	A	306.0(18.7) ^{Cb*}	A	344.4(22.7) ^{Aab}	A	363.3(25.7) ^{Ab}	A	311.0(41.8) ^{Bb*}	A	347.2(22.6) ^{Aa}	A	354.8(32.6) ^{ABa}	A
	T_4	335.5(40.0) ^{Ab*}	A	350.1(28.2) ^{ABab}	A	362.8(20.1) ^{Aa}	A	332.5(22.7) ^{Bb}	A	351.1(22.7) ^{Aab}	A	365.2(27.5) ^{Aa}	A	326.7(28.3) ^{Bb*}	A	335.9(15.9) ^{ABab}	A	354.5(20.3) ^{ABa}	A

NC: T_0: 356.9(31.7) - **T_1:** 355.6(33.0) - **T_2:** 379.5(23.3) - **T_3:** 360.5(36.4) - **T_4:** 370.9(43.8)

Distinct uppercase letters compare the means among the different time points and within the same experimental gel. Distinct lowercase letters compare the means among different % of NP (0, 5 and 10) and within the same % of HP and time point. Distinct and isolated uppercase letters (in the middle collums) indicate significant difference among the % of HP (6, 15 and 35%) within the same % of NP and time point. Asterisks indicate significant differences with the negative control (NC) within each time point separately. All the statistical tests were conducted taking into consideration a 5% level of significance

Ra

Table 2 illustrates the *Ra* values obtained before (T_0) and 14 days after bleaching (T_4). No significance was detected in any of the factors and their interactions (ANOVA; $p > 0.05$). At both time points, no differences were noted among groups or NC (Dunnet; $p > 0.05$), or T_0 and T_4 ($p > 0.05$).

Table 2. Means and standard deviation *Ra* values obtained taking into consideration the enamel surface tested before bleaching (T_0) and 14 days of storage in artificial saliva (T_4)

		HP6			HP15			HP35		
NOLT		**0NP**	**5NP**	**10NP**	**0NP**	**5NP**	**10NP**	**0NP**	**5NP**	**10NP**
	T_0	0.029 (0.005)	0.033 (0.009)	0.027 (0.005)	0.028 (0.008)	0.025 (0.007)	0.029 (0.008)	0.033 (0.009)	0.032 (0.006)	0.028 (0.012)
	T_4	0.030 (0.007)	0.032 (0.008)	0.028 (0.006)	0.030 (0.010)	0.027 (0.007)	0.029 (0.009)	0.030 (0.008)	0.031 (0.010)	0.027 (0.007)
		HP6			**HP15**			**HP35**		
LT		**0NP**	**5NP**	**10NP**	**0NP**	**5NP**	**10NP**	**0NP**	**5NP**	**10NP**
	T_0	0.033 (0.009)	0.031 (0.013)	0.027 (0.004)	0.032 (0.010)	0.026 (0.010)	0.030 (0.010)	0.032 (0.006)	0.026 (0.004)	0.030 (0.007)
	T_4	0.032 (0.005)	0.030 (0.006)	0.027 (0.006)	0.025 (0.006)	0.028 (0.004)	0.032 (0.012)	0.027 (0.007)	0.032 (0.007)	0.033 (0.006)
NC: T_0: 0.029 (0.007) – **T_4:** 0.031 (0.006)										

ΔS and Demineralization Depth

No statistically significant differences among factors or interactions were noted for ***ΔS*** ($p > 0.05$, Table 3) . Also, none of the groups presented statistical differences with the negative control ($p > 0.05$).

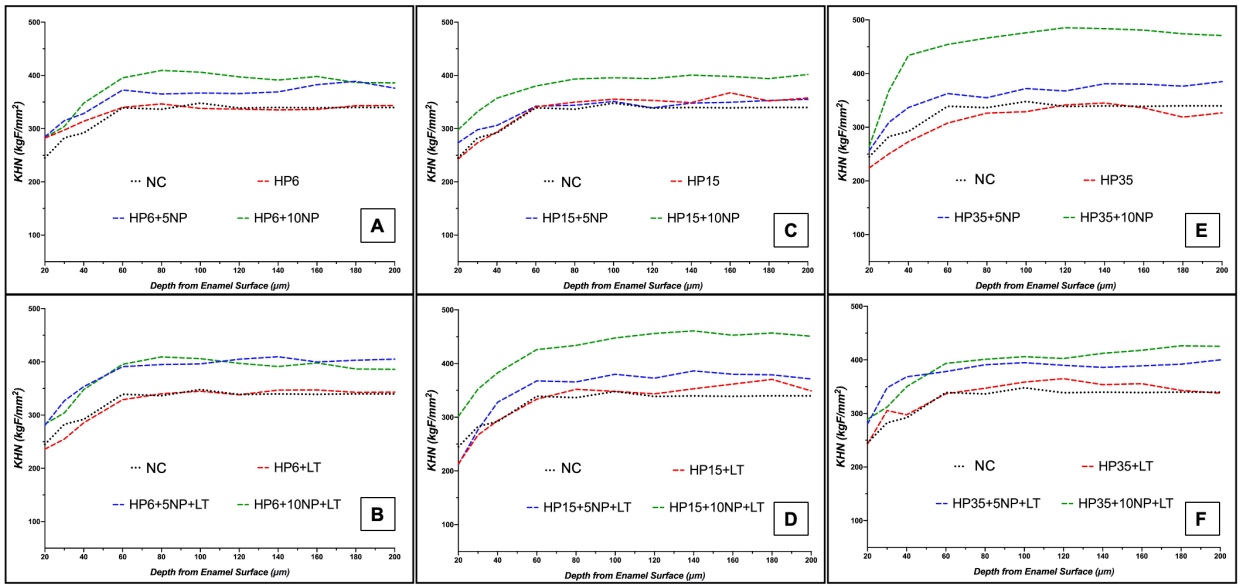
Table 3. Means and standard deviation of ΔS values calculated from the cross-sectional KHN values from 20 to 200 μm of distance from the enamel surface

		HP6	HP15	HP35
NO LT	0NP	9.597,6 (3.690,0)	10.197,4 (3.702,4)	10.288,8 (4.105,5)
	5NP	10.707,7 (1.559,5)	8.955,4 (3.935,5)	11.793,9 (3.255,7)
	10NP	10.857,5 (2.496,5)	11.438,0 (4.071,3)	12.274,8 (3.759,3)
LT	0NP	11.320,3 (3.746,6)	11.694,9 (3.219,8)	10.906,0 (4.059,5)
	5NP	12.136,2 (3.554,9)	11.860,0 (4.087,1)	10.452,5 (2.878,1)
	10NP	10.235,3 (2.173,5)	12.471,4 (2.791,9)	11.828,2 (4.695,4)
NC: 9.756,0 (2.570,6)				

Figure 1 depicts the mean cross-sectional KHN values. Overall, the non-containing NP groups (red lines) exhibited a KHN similar to the NC (black lines). In most occurrences, the 5NP (blue lines) and 10NP (green lines) groups presented curves higher than NC,

especially for 10NP-containing gels. It is possible to observe that the plateau of the curves occurred mostly at 60-μm distant from the outer enamel surface.

Figure 1. Graphs representing the KHN x μm progression for each group as compared to the negative control (NC)



The black dotted line represents the NC group, repeated over all the graphs (A-F). The red dashed lines indicate the groups treated with non-containing NP gels, while blue and red the 5NP and 10NP ones, respectively. HP6 gels are without (A) and with (B) light (LT) are illustrated in the left side, HP15 gels without (C) and with (D) LT in the center, and HP35 gels without (E) and with (F) LT in the right side.

Figure 2 reveals that enamel surface and subsurface were sound in the negative control group, with no apparent enamel diffraction. Some groups displayed darkened areas, represented by asterisks, that could be suggestive of enamel demineralization. Such regions can be observed in HP35 bleached enamel bleached, independently of the presence of NPs, and also with HP6 without NPs. All groups exhibited a surface free of major irregularities such as valleys caused by surface disruption.

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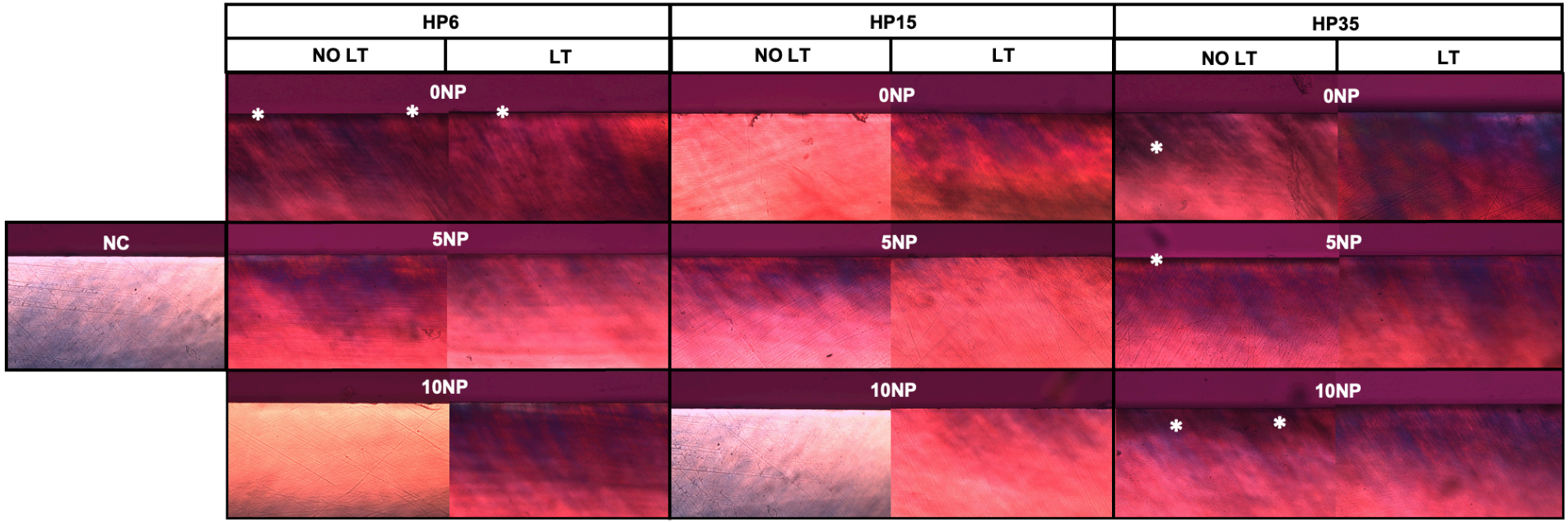
Figure 2. Representative polarized microscopy images acquired 14 days after the last bleaching session (T_4)

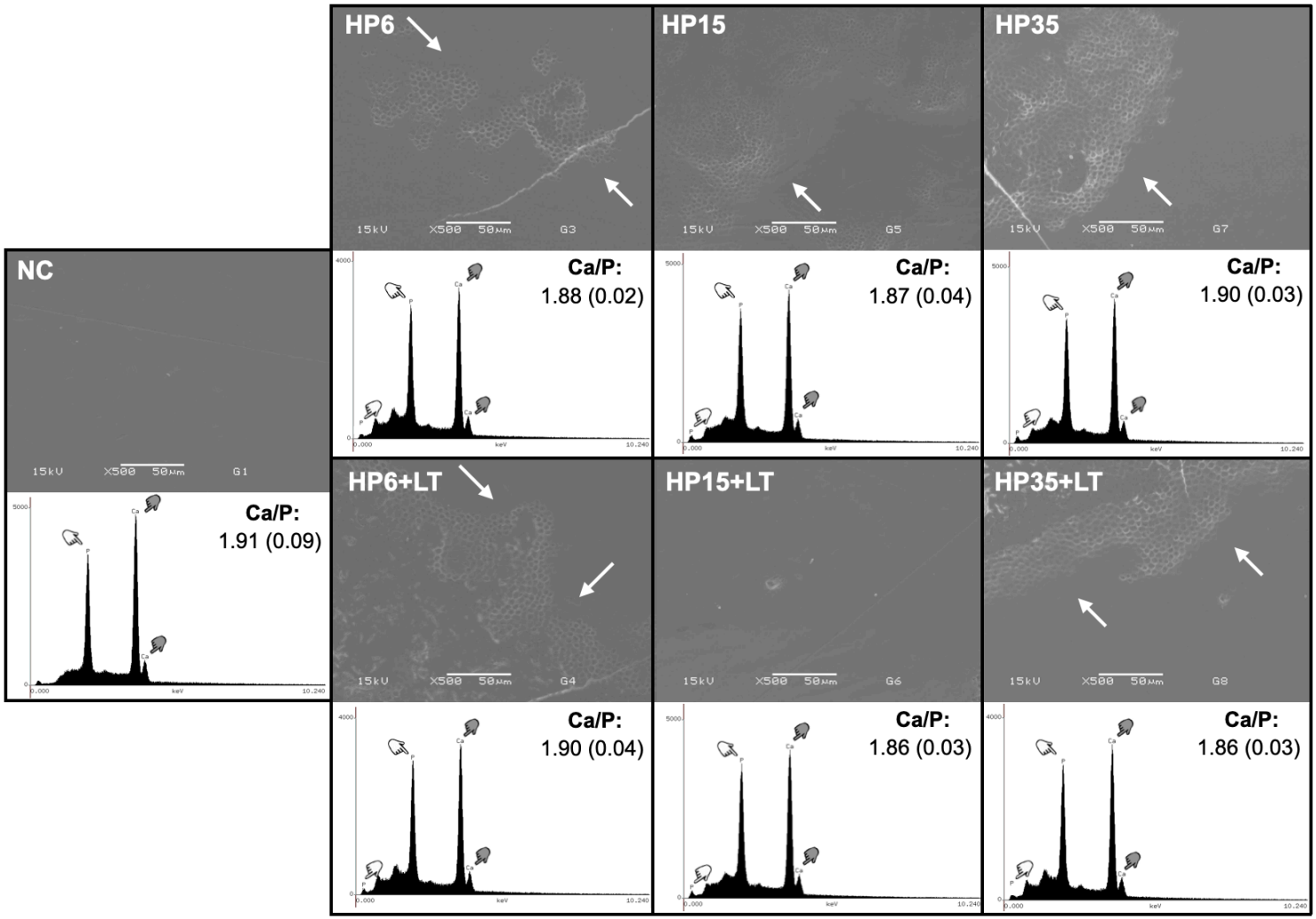


Most of the experimental groups exhibited regular surface and subsurface characteristics as comparable to the negative control (NC), Free of major irregularities and steps caused by surface disruption. The asterisks point to darkened surface areas. (HP6, HP6+LT and HP35+5NP) and in the subsurface (HP35, HP35+10NP) of enamel.

Enamel Surface Morphology and Ca/P

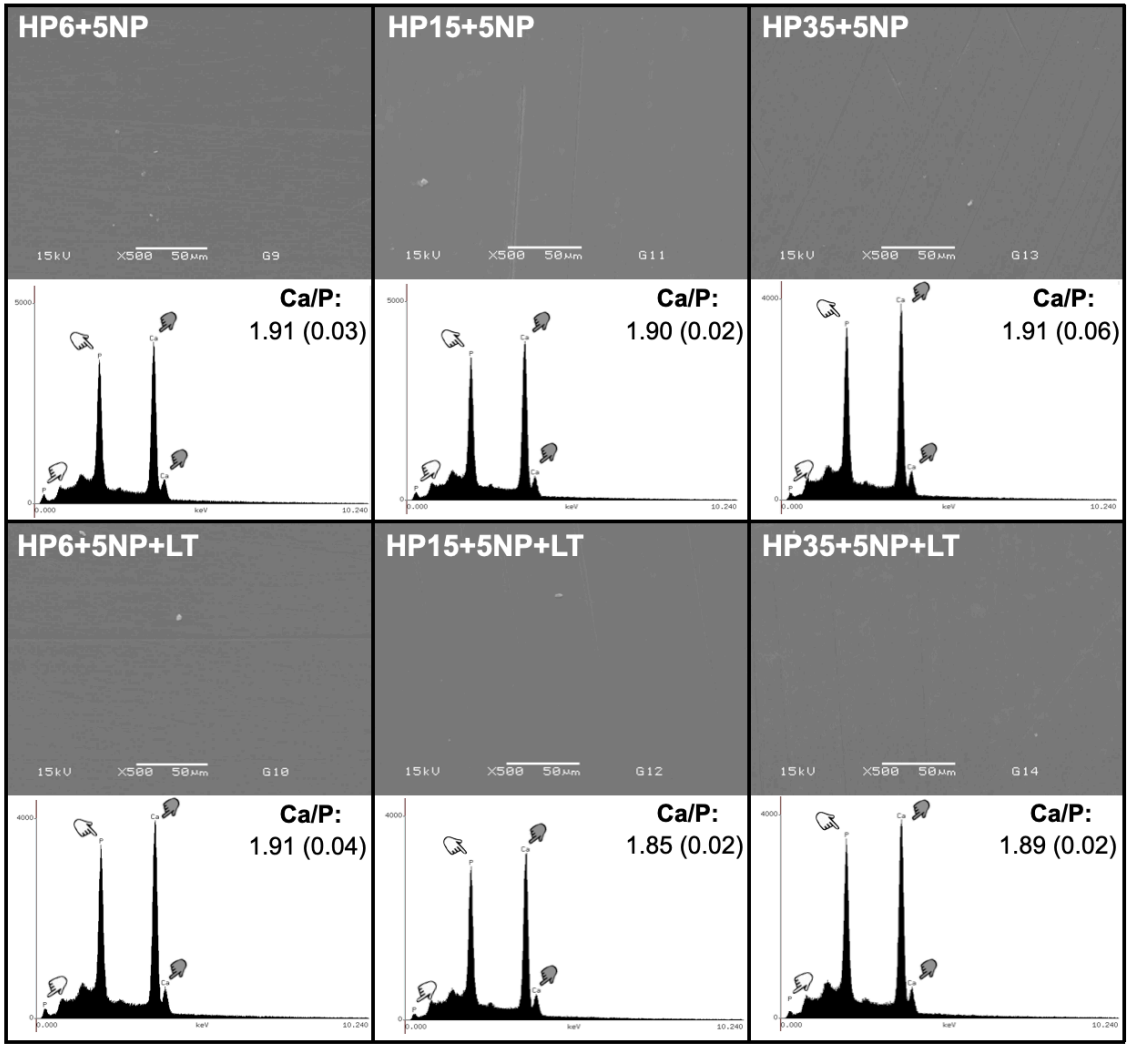
Figures 3, 4 and 5 display representative SEM images of enamel and the EDS elemental mapping for each group at T_4. NC exhibited a flat and polished surface, whereas the non-containing NP groups presented at least some extent of irregularities comparable to the removal of intraprismatic core and presence of deep porosities and pits (white arrows – Fig. 2). The groups bleached with experimental gels incorporated with 5NP (Fig. 3) and 10NP (Fig. 4) exhibited surfaces more compatible with the NC, with the continuity of the polished enamel. The semi-quantitative analysis of EDS revealed that the calcium to phosphorous ratio in the NC was equal to 1.91 ± 0.09. The Ca/P ranged from 1.86 ± 0.03 to 1.90 ± 0.04 in the non-containing NP groups. This ratio varies from 1.85 ± 0.02 to 1.91 ± 0.06 and from 2.02 ± 0.12 to 2.06 ± 0.14 in the 5NP and 10 NP groups, respectively.

Figure 3. Representative SEM images and EDS elemental mapping of negative control and non-containing NP groups obtained 14 days after the last bleaching session (T_4)



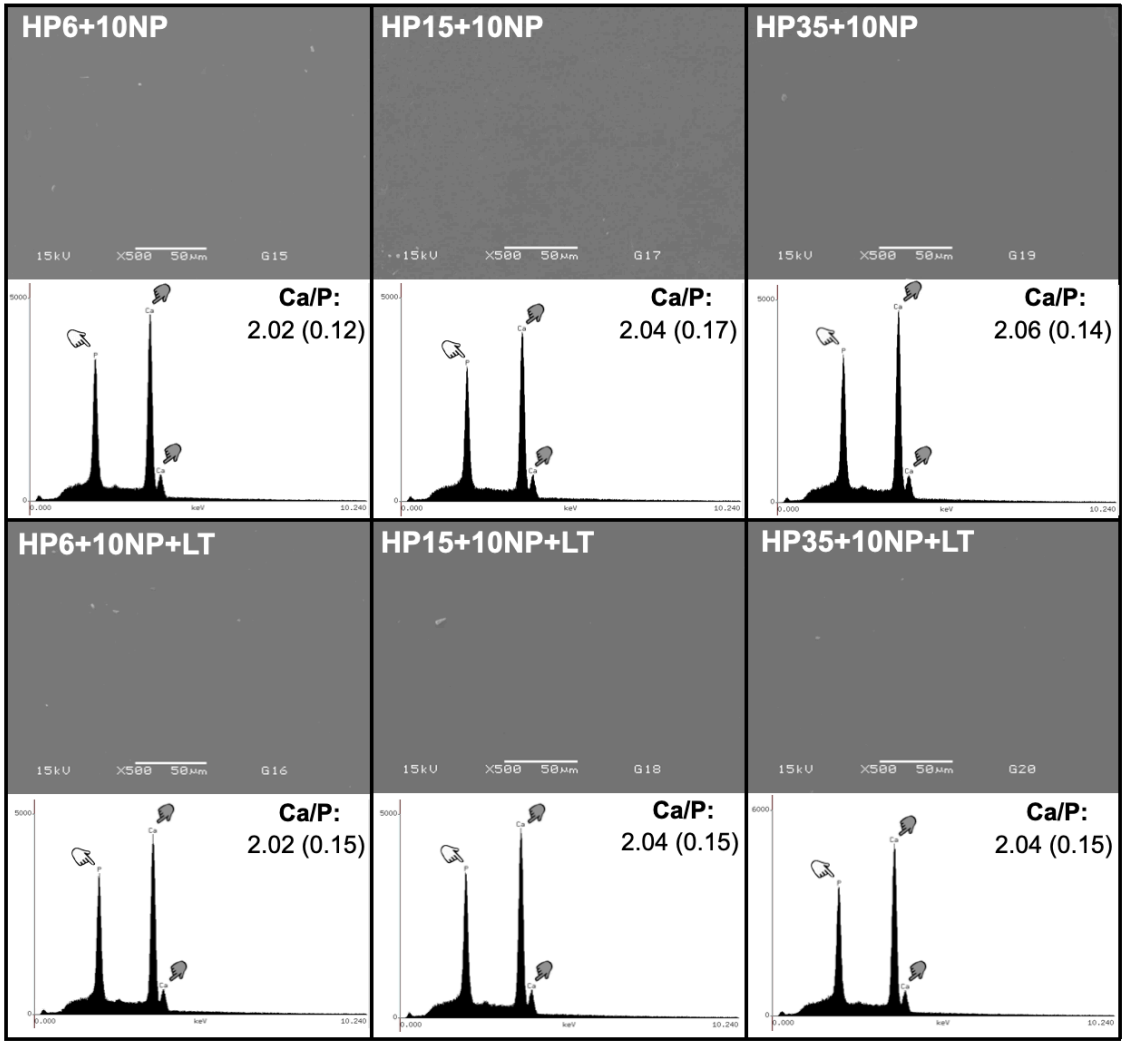
Negative control (NC) exhibited a flat and polished surface area. Most of the bleached groups herein illustrated presented discontinuity of thepolished enamel surface, as shown by the white arrows pointing to affected intraprismatic areas. The grey finger points to the peaks of the Ca element, and the white finger corresponds to the P peaks in the elemental mapping images

Figure 4. Representative SEM images and EDS elemental mapping of negative control and 5NP groups obtained 14 days after the last bleaching session (T_4)



The 5NP groups maintained the continuity of the enamel surface, exhibiting a flat and homogenous area. Some marks in the surface (HP15 and HP6+5NP+LT) could be a result from the polishing procedures. The surface characteristics are similar to those found in the NC groups seen in Fig. 2. The grey finger points to the peaks of the Ca element, and the white finger corresponds to the P peaks in the elemental mapping images.

Figure 5. Representative SEM images and EDS elemental mapping of negative control and 10NP groups obtained 14 days after the last bleaching session (T_4)



The 10NP groups exhibited a flat and homogenous area, with the continuity of the enamel surface maintained. As for the 5NP groups, the surface characteristics detected in the NC group (Fig. 2) were maintained. The grey finger points to the peaks of the Ca element, and the white finger corresponds to the P peaks in the elemental mapping images.

DISCUSSION

The microhardness assessment was used by several other investigations to indirectly indicate whether the proposed treatments resulted in enamel demineralization or not [13, 14, 25, 28, 30]. Based on the results, it could be assumed that the experimental HP6, HP15 and HP35 gels without the NP (light-irradiated or not) were not capable of upholding the enamel mineralization levels, 24 h elapsed from the first bleaching session to the end of the study. Even though most of these groups partially recovered the reduction in KHN after 14 days in artificial saliva immersion, all of them were still significantly inferior to the negative control. On the other hand, all the NP-incorporated gels (with or without LT) remained similar to the NC. Therefore, the first null hypothesis was accepted since the co-doped titanium dioxide nanoparticles did not affect the enamel microhardness.

Others have shown that commercial and experimental gels with acidic and even neutral pH significantly reduces enamel surface microhardness immediately after the gel's application [32-34]. Contrarily to these findings, Parreiras et al. (2014) [32] reported a significant KHN recovery after storage in artificial saliva for 7 days, reaching the baseline values, which was not the case in this study. The pH of the experimental HP gels herein tested was previously reported [21], indicating that the pH of carbomer-based polymer ranged from 5.1 (HP6) to 5.5 (HP15 and HP35) when mixed with HP solutions.

One might say that the acidic pH of the resulting gels could have played the main role in this scenario, but in a recent study, Torres et al. (2022) observed that an experimental carbomer-based bleaching gel adjusted to pH 6.5 promoted a significant enamel microhardness reduction, leading to the highest decrease detected among all the tested thickeners (carbomer, modified sulfonic acid, alkali swellable emulsion, semi-synthetic polysaccharide, and particulate colloids) [35]. Also, an *in situ* study demonstrated that a neutral carbomer gel without HP downgraded enamel surface microhardness [36], and there is evidence that polyacrylate-based polymers inhibited the growth of hydroxyapatite crystals [37]. According to Torres et al. (2022), a feasible explanation for this phenomenon, relies on the Ca^{++} ions released from the enamel permeating the gel and complexing with the anions in the carbomer, turning the polymer undersatured [35].

Nonetheless, the pH of the polyacrylate-based gels should not be ruled out as an important protective factor to the enamel surface microhardness, once the co-doped titanium dioxide incorporation into these gels increased their pH up to 6.0, showing a slightly higher increase after the 10NP addition [21]. In this study, the experimental gels incorporated with 5% and 10% nanoparticles prevented a significant decrease in surface microhardness. It is also fundamental to bear in mind that the NP composition could have influenced this scenario. The fluorine content in the crystal lattice of the co-doped nanoparticle could have potentially favored the formation of fluorapatite based on an ion-exchange mechanism, turning the enamel surface more resistant to eventual pH drops [38] and undersaturation of the polymeric network in the gels. Indeed, other studies have already attested that incorporation of fluoride ions into bleaching gels upheld the microhardness, which was not observed in the fluoride-free gels [10, 34]. However, this aspect should be further investigated as the concentration of fluorine in the experimental gel's environment could not suffice for such event to happen, and some studies have also indicated that incorporation of the calcium ion could offer increased microhardness protection when compared to fluorine [39, 40].

A previous FTIR analysis of dental enamel submitted to the gels tested herein has demonstrated a protection trend favored by NP on the mineral ratio of carbonate to phosphate peaks, and a preservation of these peaks' shape and absorbance values was also detected in comparison to the expressive changes caused by the carbomer-based gels without NP [21]. The decrease in the enamel mineral ratio has already been demonstrated along with a significant reduction in the enamel surface microhardness [41], which was corroborated by the present findings. Moreover, the present energy dispersive x-ray spectroscopy revealed a numerically higher Ca/P ratio for groups bleached with 10NP. Yet, the EDS analysis is a semi-quantitative and sub-surface analysis [42] and that should be inferred with caution, most importantly because it was performed in a small number of specimens (n = 2/group).

Despite the surface loss detected in groups bleached without the NP, the in-depth microhardness analysis concluded that none of the bleaching protocols negatively affected the cross-sectional microhardness in comparison with the negative control. The mean ΔS detected in the groups are in accordance with those reported for sound dental

enamel [30]. The lower values of KHN up to 60 μm in depth was detected in previous studies after different types of surface treatments, even when the surface was left untreated [30, 34, 43]. Thus, it could be inferred that outermost layers of the subsurface holds lower microhardness, thereby justifying the ΔS results herein found. In the present study, the conversion of microhardness to mineral concentration was not performed because of the formula's discrepancy in the literature [44, 45]. Nevertheless, both studies [44, 45] concluded that microhardness could be used as a measure of alterations in mineral content in *in vitro* studies, after a significant and direct co-relation with transversal micro radiographical data. It is noteworthy that the in-depth KHN profiles for the groups bleached with NP, especially those with 10NP, were higher than their corresponding NP-free gels and NC, even in the outermost layers of the subsurface (up to 60 μm). Likewise, Cavalli et al. (2012) have found a similar pattern for the enamel mineral volume concentration in function of the lesion depth when comparing carbomer-based gels with or without sodium fluoride incorporation [11]. Therefore, the fluorine present in the crystal lattice of the NP could have also influenced the enamel cross-sectional microhardness.

Interestingly, the polarized light microscopy indicated some darkened areas in the subsurface of some groups, which could emphasize that surface of enamel is dynamic with some areas presenting higher mineral content than others. Previous studies also found some darkened areas indicative of minor demineralization in groups with significantly higher maintenance of the mineral content than others [10, 11, 46]. Based on the low number of specimens tested under PLM (two 100-μm thick enamel slice), these images are only representative. Further studies should increase the sample size and/or amplify the regions evaluated within the same specimens to successfully co-relate these outcomes to those found under the cross-sectional microhardness.

Furthermore, the enamel surface roughness was maintained similar after the bleaching performances, accepting the second and last null hypothesis. A previous observation of the *Ra* values under atomic force microscopy [21] indicated that some specimens treated with NP gels had higher *Ra* than others, but the statistical results from the present profilometry method pointed out that the experimental gels would not affect the roughness profile of enamel, as stated for other available in-office bleaching protocols [47]. It is important to highlight that the isolated affected intraprismatic areas detected

under SEM observation did not influence the *Ra* values of the groups bleached without NP. These findings are different from those reported by Wijetunga et al. (2021), who showed that an acidic carboxymethyl cellulose-based bleaching gel negatively influence both the *Ra* and the surface morphology [48]. Either way, previous studies have also illustrated some changes in the surface morphology when treated with commercial carbomer-based hydrogen peroxide bleaching gels, but the alterations (fissures, pits and deep valleys) seemed to be more homogenous and to cover the entire surface under the SEM [26, 31, 49] in contrast to the punctual ones observed in the present study.

Hence, the evaluation of the various enamel surface aspects herein performed strongly indicates that the use of the experimental gels would not harm the dental enamel, but the incorporation of the NP could prevent the surface from microhardness loss and morphology alterations. The evaluation of three different HP concentrations under dark and light conditions took place because the incorporation of NP was shown to increase the HP6 and HP15 efficacy as comparable to HP35 [21]. However, the incorporation of 5NP into HP6, followed by LT irradiation, was sufficient to overcome the bleaching outcomes of HP35. The incorporation of other chemical photocatalysts into bleaching gels are also accountable for the increase in their efficacy [18, 19, 50], which highlights the increasingly search for alternatives towards safer in-office bleaching therapies.

The present follow-on assessments, then, suggest that co-doped titanium dioxide nanoparticles would not only allow the reduction of HP's concentration up to 5 times, as stated previously [21], but it also could protect the enamel surface when incorporated into a carbomer-based polymer. The dramatic reduction in the HP concentration is likely to be directly related to the lower diffusion of reactive oxygen species into the pulp chamber, thereby lowering the chances of cell damage and oxidative stress. Consequently, this approach holds an in-office bleaching protocol promise with reduced risk of tooth sensitivity but still ensuring optimal and highly satisfactory esthetic outcomes, not affecting the dental enamel physical properties.

CONCLUSION

Within the limitation of the present study, the following conclusions could be drawn:

- The lack of NP in the experimental gels significantly reduced the surface microhardness. However, the experimental carbomer-based gels incorporated with the co-doped TiO_2 nanoparticles preserved the enamel surface microhardness throughout the entire in-office bleaching protocol, regardless of the HP concentration and LT irradiation.
- None of the experimental bleaching gels significantly affected the enamel cross-sectional microhardness and its surface roughness.

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2.5 Artigo 5. Co-doped titanium dioxide nanoparticles decrease the cytotoxicity of experimental hydrogen peroxide gels for in-office tooth bleaching

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ABSTRACT

Objective: To evaluate the efficacy and cytotoxicity of experimental 6% and 35% hydrogen peroxide gels (HP6 or HP35) incorporated with titanium dioxide nanoparticles (NP) co-doped with nitrogen and fluorine and irradiated with a violet LED light (LT).

Methods: Bovine enamel-dentin disks adapted to artificial pulp chambers were randomly assigned to bleaching with (n=8/group): NC (negative control), NP, HP6, HP6+LT, HP6+NP, HP6+NP+LT, HP35, HP35+LT, HP35+NP, HP35+NP+LT, and commercial HP35 gel (COM). Color (ΔE_{00}) and whiteness index (ΔWI_D) changes were measured before and 14 days after bleaching. The extracts (culture medium + diffused gel components) collected after the first session were applied to odontoblast-like MDPC-23 cells, which were assessed concerning their viability, oxidative stress, and morphology. The amount of HP diffused through the disks was determined. Data were analyzed by one-way ANOVA/Tukey or Welch's ANOVA/Games-Howell Tests (α=5%).

Results: HP6+LT, HP6+NP, HP6+NP+LT, and HP35 groups promoted similar ΔE_{00} and ΔWI_D ($p>0.05$). HP6+NP+LT exhibited ΔWI_D higher than HP6 ($p<0.05$), the lowest HP diffusion, and the highest cell viability (%) among bleached groups, preserving cell morphology and number of living cells similar to NC and NP. HP6+LT, HP6+NP, and HP6+NP+LT exhibited the lowest cell oxidative stress among bleached groups ($p<0.05$). HP35, HP35+LT, and HP35 (COM) displayed the lowest cell viability.

Significance: Incorporation of the nanoparticles not only increased the efficacy of HP6 gel with light, but also reduced the cytotoxicity caused by the standard in-office bleaching gel concentration. This approach holds the promise to clinically render optimal esthetic outcomes while potentially reducing the tooth sensitivity.

Key Words: Tooth Bleaching, Hydrogen Peroxide, Light Irradiation, Nanoparticles, Toxicity.

INTRODUCTION

In-office tooth bleaching, a procedure widely performed in dental offices, holds the advantages of being a low-cost and ultraconservative approach in comparison to restorative treatments [1]. In this procedure, tooth discoloration is resolved by the application of hydrogen peroxide (H_2O_2) gels at high concentrations (25-40%) onto the buccal surface of dental enamel [2, 3]. The low molecular weight of H_2O_2 allows its diffusion through the interprismatic spaces of the enamel towards the dentin, where reactive oxygen species (ROS) generated by decomposition of H_2O_2 interact with and break down staining structures, the so-called chromophores. Consequently, teeth look whiter [1, 4].

In spite of the optimal esthetic outcomes attained with high-concentrated H_2O_2 gels [3], several studies proved that standard in-office protocols may negatively influence the enamel surface properties [5-7]. These professional protocols have also caused undesirable cytotoxic effects in an application time- and concentration-dependent relation [8, 9] due to the trans-amelodentinal diffusion of high concentration of H_2O_2 to reach pulp chamber. From the clinical standpoint, bleaching protocols with such high H_2O_2 concentrations are expected to cause higher risk and intensity levels of tooth sensitivity [10, 11], and their long-term effects on the pulp structure remain unknown. A previous report demonstrated that reducing the concentration of H_2O_2 in the bleaching gel and shortening its application time up to three times significantly protected the cell viability, but the esthetic outcome was compromised [8].

In this critical context, recent efforts such as the incorporation of catalysts (i.e., titanium dioxide [TiO_2] and manganese oxide [12, 13]) into low-concentrated experimental gels have attempted to counterbalance the decrease in the cytotoxicity of protocols with the maintenance of the highly effective esthetic outcomes by accelerating the decomposition of H_2O_2 into ROS. Also, reports have attested to the significant increase in the efficacy of 6 to 15% hydrogen and 37% carbamide peroxide gels when irradiated with a novel generation of violet LED light for in-office tooth bleaching [10, 14].

Recently, an *in vitro* study successfully incorporated for the first time TiO_2 nanoparticles co-doped with nitrogen and fluorine into experimental 6% and 15% hydrogen peroxide gels used for in-office bleaching [15]. The co-doped TiO_2 nanoparticles

have been previously synthesized by means of robust solvothermal reactions, resulting in the stable anatase phase of TiO_2 and the generation of long-living ROS species [16]. The authors showed that the combination of the co-doped TiO_2 nanoparticles in the 6% gel with violet LED irradiation upheld esthetic outcomes similar to those of 35% hydrogen peroxide gel [15]. In addition, the experimental gels containing the co-doped TiO_2 nanoparticles presented a higher pH, maintained the enamel surface topography, and preserved the carbonate and phosphate levels on enamel in comparison to nanoparticle-free gels [15]. These results are promising to achieve clinical success, which was not observed in a previous clinical trial that incorporated commercially available TiO_2 nanoparticles into a 6% hydrogen peroxide gel without achieving the efficacy of 35% gel [17].

Because the co-doped TiO_2 nanoparticles have been shown to optimize the dissociation of H_2O_2 dissociation into long-lived ROS [18], it is expected that longer interaction time between ROS and organic chromophores would lead to better esthetic outcomes and lower levels of cytotoxicity that precipitates from unreacted H_2O_2 by-products. Therefore, low-concentrated experimental gels containing these nanoparticles could allow for an improved esthetic and reduced cytotoxicity effects following in-office tooth bleaching. Based on context provided, the present study aimed to evaluate the effects of these novel gels on the bleaching efficacy, on amounts of diffused H_2O_2, and on the viability of odontoblast-like cells. The null hypotheses were that the nanoparticle incorporation i) would not improve the efficacy of in-office bleaching gels and ii) would not increase the cytotoxicity of the proposed treatments.

MATERIAL AND METHODS

Experimental Design. Enamel-dentin disks (n=8/group) were submitted to bleaching with 6% and 35% hydrogen peroxide experimental gels (HP). These gels were incorporated or not with TiO_2 nanoparticles (NP) co-coped with nitrogen and fluorine and irradiated or not with a violet LED light (LT). A commercial 35% HP gel (HP COM) and an experimental gel only with NP were also used. The negative control (NC) did not receive any bleaching treatment. The color change (ΔE_{00}) and whitening effect (ΔWI_D) were assessed before the bleaching procedures (T_0) and 14 days after the last session (T_4). The trans-amelodentinal hydrogen peroxide diffusion and cytotoxicity (cell viability, oxidative stress, cell morphology, and Live/dead assay) were measured at the first bleaching session (T_0).

Specimens' Preparation and Group Distribution. Intact bovine incisors were extracted, cleaned, and stored at 4ºC for no longer than 30 days. Eighty-eight enamel-dentin disks were obtained from the middle third of the incisor's buccal surface using a diamond bur for glass (⌀8mm, Di Martino Brocas Diamantadas Ltda, Campinas, SP, Brazil) coupled to a bench drill (Pratika FSB16P, Schultz, Joinville, SC, Brazil). The diameter of the disks was 5.6mm and the thickness (enamel-dentin) was standardized at 2.3 ± 0.2 mm using 400-grid sandpapers (3M Brasil, Sumaré, SP, Brazil). Then, the disks were positioned in a white and opaque tile to allow the colorimetric measurement with a hand-held spectrophotometer (Easy Shade,Vita Zahnfabrik, Bad Sackingen, Germany). The coordinate values (L*, a*, b*, h, and C) were used to randomly allocate the specimens into 11 groups. One-way ANOVA test confirmed that no significant differences were found among the groups regarding the color coordinates ($p>0.05$). The groups were randomly assigned to each proposed bleaching protocol, as follows:

- NC: negative control – without treatment;
- NP: experimental bleaching gel only incorporated with the co-doped nanoparticles;
- HP6: experimental 6% hydrogen peroxide gel;
- HP6+LT: experimental 6% hydrogen peroxide gel irradiated with violet LED light;
- HP6+NP: experimental 6% hydrogen peroxide gel incorporated with NP;
- HP6+NP+LT: experimental 6% hydrogen peroxide gel incorporated with NP and light-irradiated;

- HP35: experimental 35% hydrogen peroxide gel;
- HP35+LT: experimental 35% hydrogen peroxide gel irradiated with violet LED light;
- HP35+NP: experimental 35% hydrogen peroxide gel incorporated with NP;
- HP35+NP+LT: experimental 35% hydrogen peroxide gel incorporated with NP and light-irradiated;
- HP35 (COM): commercial 35% hydrogen peroxide gel (Whiteness HP, FGM, Joinville, SC, Brazil).

Nanoparticles' Synthesis. The synthesis of the co-doped titanium dioxide nanoparticles has been reported in previous publications [15, 16]. Briefly, 1.7 g of Ti $(OBu)_4$ (Aldrich, 97%), 4.6 g C_2H_5OH (200-proof Decon Labs, King of Prussia, PA, USA), 6.8 g $C_{18}H_{35}NH_2$ (Aldrich, 70%), 7.1 g $C_{18}H_{34}O_2$ (Aldrich, 90%) and 5% of NH_4F (based on Ti content; crystalline, ACS, Alfa Aesar) were mixed with an ethanol-water solution (4%, 18-Milli-Q; total weight = 13.10 g). The final solution was dispensed into a high-pressure reaction vessel (Borosilicate Glass-lined; Paar Series 4593, Bench Top Reactor System, Moline, Il, USA), reacted (180 °C, 24 hours, 15 psi), and stirred (280 rpm) during 24h. Following this cycle, the solution was transferred to a falcon tube with ethanol (200-proof, Decon Labs, King of Prussia, PA, USA) and centrifuged for 15 min at 8,000 rpm. This procedure was repeated two additional times, using 20 mL of ethanol.

Experimental Gel's Synthesis and Incorporation of NPs. The synthesis of the experimental gel was previously described [15]. A commercially available hydrophilic polymer (Carbomer 940 NF, Spectrum, Gardena, CA) was mixed within ultrapure water using a planetary and orbital stand-alone mixer (Speed Mixer, DAC 400.1 FVZ, FlackTek Inc, Laudrum, SC, USA). The pH of the resulting gel was around 6.0. One-mL aliquots of the co-doped nanoparticles (NP, ~ 40 mg/mL) suspended in ethanol were transferred to separated falcon tubes and were centrifuged at 8,000 rpm for 5 min. The ethanol was removed from the tube and the NPs were incorporated into 20 g of the experimental gels, which were mixed at 2,450 rpm for 20 s (Speed Mixer, DAC Iso.1 FVZ, FlackTek Inc, Laudrum, SC, USA). As a result, the gels contained 5% of NP.

Bleaching Procedures. The gels (with or without the incorporation of NP) were manually mixed with stock 6% or 35% HP solutions following the previously published protocol [15]. At the first bleaching session (T_1), 20 μL of the commercial or each experimental gel was applied onto the buccal enamel surface using a viscosity pipette. The gels remained in contact with the surface for 30 min without renewal. Light-irradiated groups (LT) received twenty 1-min irradiations with consecutive 30-s intervals from a violet LED light (Bright Max Whitening, MMOptics, São Carlos, SP, Brazil) [19]. The light unit was positioned 8 mm away from the buccal surface of the specimens. The same procedures were repeated at the second (T_2) and third (T_3) sessions. The specimens were stored in 100% humidity at 37ºC [20] among the sessions and for 14 days (T_4) after the last bleaching session.

Colorimetric Evaluation. At T_0 and T_4, the enamel-dentin disks were positioned in the white and opaque tile to allow the measurement of the color coordinates with the spectrophotometer. The color change (ΔE_{00}, equation 1) was calculated with the CIEDE2000 system formula, taking into consideration the L*, a*, b*, h, and C values collected at T_0 and T_4. The whiteness change was calculated with the whiteness index for dentistry (WI_D, equation 2) [21]. The ΔWI_D was calculated subtracting the WI_D values (T_4 - T_0).

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + RT \cdot \left(\frac{\Delta C'}{K_C S_C}\right) \cdot \left(\frac{\Delta H'}{K_H S_H}\right)} \quad \text{(equation 1)}$$

$$WI_D = 0.55L^* - 2.32a^* - 1.100b^* \quad \text{(equation 2)}$$

Trans-amelodentinal Diffusion

Experimental Procedure. The enamel-dentin disks (n=8/group) were inserted into artificial pulp chambers (APC) illustrated in Figure 1. The disks were adapted with two silicon rings and the edges of the disks were sealed with utility wax (Cera 7 Rosa Wilson, Polidental, Cotia, SP, Brazil). Following the disk/APC sterilization with ethylene oxide (Acecil, Central de Esterilização Comércio e Indústria, Campinas, SP, Brazil), the sets were positioned in 24-well plates (KASVI, São José dos Pinhais, PR, Brazil) in contact with 1 mL of DMEM without FBS. The bleaching procedures were then executed, and the

extracts containing the DMEM and the bleaching gel components that diffused through the disks were collected and homogenized. These extracts were split into 100-μL aliquots to run the next tests described.

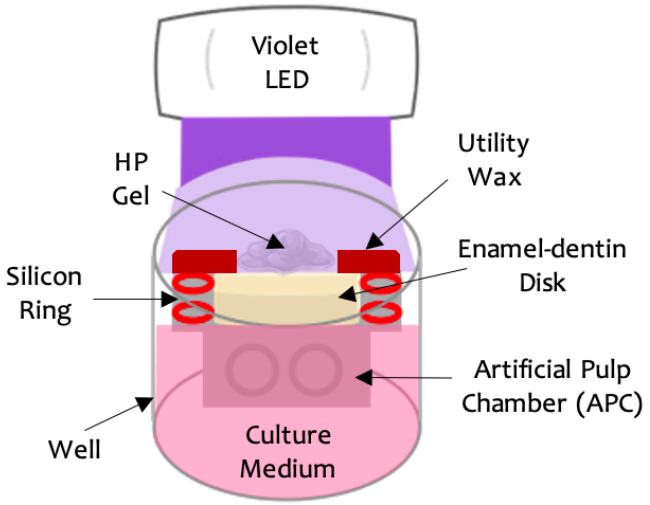

Figure 1. Illustration of the experimental procedure using the enamel-dentin disk set

Quantification of H_2O_2. An aliquot of 100 μL of the extracts was dispensed in 96-well plates with 900 μL of 2mol/M acetate buffer (pH = 4.5). This solution (500 μL) was collected and placed in tubes with 100 μL of 0.5 mg/mL leucocrystal violet reagent (Sigma-Aldrich, St. Louis, MI, USA), 50 μL of 1 mg/mL horseradish peroxidase enzyme solution (Sigma-Aldrich) and 2750 mL of distilled water. The plates were then transferred to a spectrophotometer (λ = 596 nm, Synergy H1, Biotek Instruments, Winooski, VT, USA) for measuring the absorbance of the resulting solutions. A standard curve with known concentrations of H_2O_2 was obtained to allow the optical density values conversion into 100 μg of H_2O_2 per mL of extract [13].

Trans-amelodentinal Cytotoxicity

Cell Cultivation. This study used immortalized odontoblast-like MDPC-23 cells cultivated in 24- and 96-well plates (KASVI, Curitiba, PR, Brazil) with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS;

Gibco, Grand Island, NY, USA), 2 mmol/L of glutamine (Gibco), 100 IU/mL of penicillin and 100 g/ of streptomycin (Gibco).

Cell Viability. For this test, 10% Alamar Blue (Life Technologies; Grand Island, NY, USA) solution was prepared in DMEM without FBS. Then, 500 μL was distributed to each well containing the extracts and the plates were incubated with 5% CO_2 at 37^0C for 4 h. The oxidized form of Alamar Blue presents a blue color, which is converted into its reduced form (a pink color) due to mitochondrial activity. Two hundred μL of each sample (extract) was transferred to a 96-well plate in order to determine the mitochondrial activity by measuring the fluorescence of the reduced salt at a spectrophotometer (excitation at 530-560 nm; emission at 590 nm, Synergy H1) [22]. These fluorescence values were converted into percentages by normalization using the mean of the negative control (NC) group.

Oxidative Stress. The intracellular oxygen reactive species from the cell were measured using the carboxy-H_2DCFDA fluorescent probe (Invitrogen, San Francisco, CA, USA). The probe was dispensed at a 5mM concentration for 30 min (37^0C) in 96-well plates containing the cultivated cells exposed to the extracts. The fluorescence was evaluated immediately after 30 min (429-mm excitation and 518-nm emission, Synergy H1), and the values were also normalized using the mean of NC [13, 22].

Live/Dead Evaluation. The Live/Dead Cell Viability/Cytotoxicity kit (Invitrogen) was used to qualitatively evaluate the Live/Dead expression of the cells (n = 4/group). In this method, the Ethyl1 homodimer-1 (EthD-1) fluorescence (red signal) probe binds the DNA bands solely to cells whose membranes are ruptured. The second probe (green signal), named Calcein AM (CA), is hydrolyzed with esterases present in the cytoplasm in viable cells. The bottom of the wells was analyzed using fluorescence microscopy (20x; Leica DM 5500B, Nussloch GmbH, Nussloch, Germany) [13, 22, 23].

Cell Morphology. The cells were seeded on glass slides sitting at 24-well plates (n = 2/group). The cells were fixed using 2.5% glutaraldehyde (Sigma-Aldrich) and post-fixed

with 1% osmium tetroxide (Sigma-Aldrich). Then, the cells were dehydrated with alcohol in decreasing concentrations (30, 50, 70, 90, and 100%) and chemically treated with HMDS (1,1,1,3,3,3-hexamethyldisilazane, Sigma-Aldrich). After being desiccated for at least 72 h, the glass slides were submitted to sputter coating with gold and analyzed under scanning electron microscopy (SEM, JEOL-JSM, 6460LV, Tokyo, Japan) at 200 and 500 times magnification [8].

Statistical Analyses. The collected data were explored and submitted to the evaluation of the normality and homoscedasticity assumptions. The values not meeting the normality (Shapiro-Wilk; $p < 0.05$) were transformed into Log. ΔE_{00}, $\Delta WI_{D,}$ and % of cell viability was submitted to one-way ANOVA and Tukey's test. Quantification of H_2O_2 (μg/mL) and oxidative stress values were considered normal but not homoscedastic (Levene; $p < 0.05$). Then, these data were submitted to Welch's ANOVA followed by post hoc Games-Howell Tests. The significant levels were all set at 5%.

RESULTS

Bleaching Efficacy. Figure 2 reveals that the bleaching protocols promoted significant differences in terms of color (ΔE_{00}, Fig. 2A) and whiteness index change (ΔWI_D, Fig. 2B) ($p < 0.001$). No differences in ΔE_{00} were detected among NC, NP, and HP6 ($p > 0.05$), while the ΔWI_D of NC and NP were negative and significantly lower than all the bleached groups ($p < 0.05$). No significant differences in ΔE_{00} and ΔWI_D were observed among groups bleached with 35% hydrogen peroxide, but HP35+NP and HP35+NP+LT exhibited higher ΔWI_D than HP6. In addition, no differences were observed among HP6+LT, HP6+NP, and HP6+NP+LT and the HP35 bleaching protocols. HP6+NP+LT displayed ΔWID significantly higher than HP6 ($p = 0.035$) but no different than all the groups modulated by HP35 gels ($p < 0.05$).

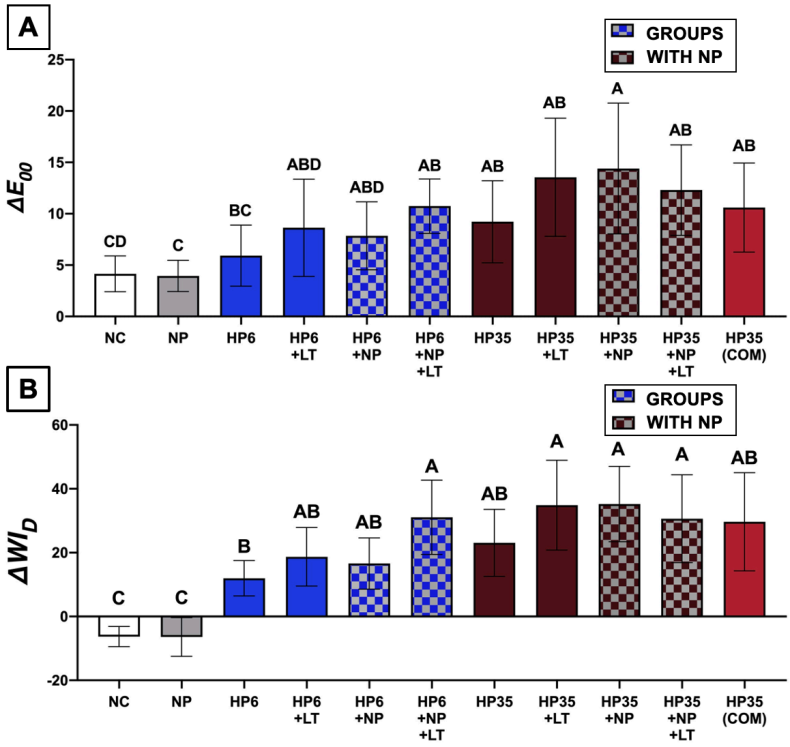

Figure 2. Mean and standard deviation ΔE_{00} (A) and ΔWI_D (B) considering the values collected before bleaching (T_0) and 14 days after the last bleaching session (T_4). Different letters indicate statistical differences, according to one-way ANOVA and Tukey's test.

Quantification of H_2O_2. Figure 3 displays the trans-amelodentinal diffusion of H_2O_2 (μg/mL) at the first bleaching application. HP35 and HP35 (COM) exhibited the highest H_2O_2 (μg/mL) values ($p < 0.05$) among groups. Within the groups treated with experimental HP35 gels, the quantification of H_2O_2 was ranked by irradiation of LT, presence of NP, and NP + LT combination (HP35 > HP35+LT > HP35NP > HP35+NP+LT; $p < 0.05$). Bleaching with experimental HP6 gels significantly reduced the trans-amelodentinal diffusion of H_2O_2 in comparison to the HP35 gels, and the HP6+NP+LT protocol further decreased H_2O_2 values ($p < 0.05$).

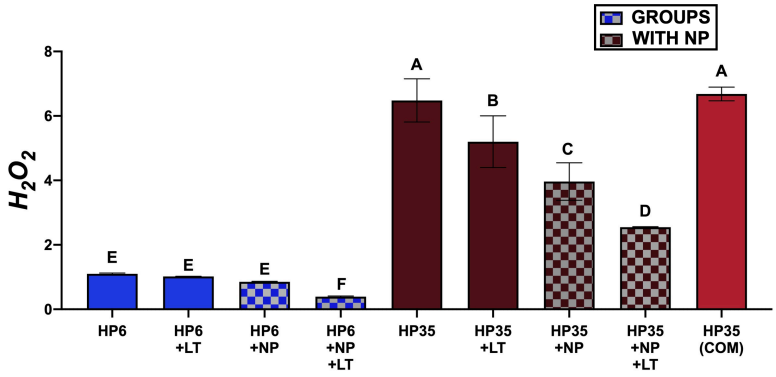

Figure 3. Mean and standard deviation values of the H_2O_2 diffusion (μg/mL) obtained at the first bleaching session (T_1). Different letters indicate statistical differences, according to one-way ANOVA and Tukey's test.

Cytotoxicity. Figure 4 demonstrates that the different bleaching protocols significantly influenced the cell viability (Fig. 4A) and oxidative stress (Fig. 4B) ($p < 0.001$). NC and NP ($p = 0.999$) maintained the MDPC-23 cell viability at 100% (Figure 4A). Experimental HP35, HP35+LT, and HP35 (COM) caused the lowest percentage [12.5 (4.8) to 14.4 (2.6)] of viable cells (Fig. 4A) and the highest oxidative stress [4.8 (0.5) to 5.0 (0.4)] levels ($p < 0.05$, Fig 4B). Among the high-concentrated gels, cells in HP35+NP+LT exhibited higher viability (Fig 4B) and lower oxidative stress (Fig 4B) than cells in HP35 (experimental and commercial), HP+LT, and HP35+NP gels ($p < 0.05$). Among groups, the highest percentage of cell viability was observed in HP6+NP+LT ($p < 0.05$, Fig. 4A), in which the oxidate stress rate was compatible with the NC and NP control groups.

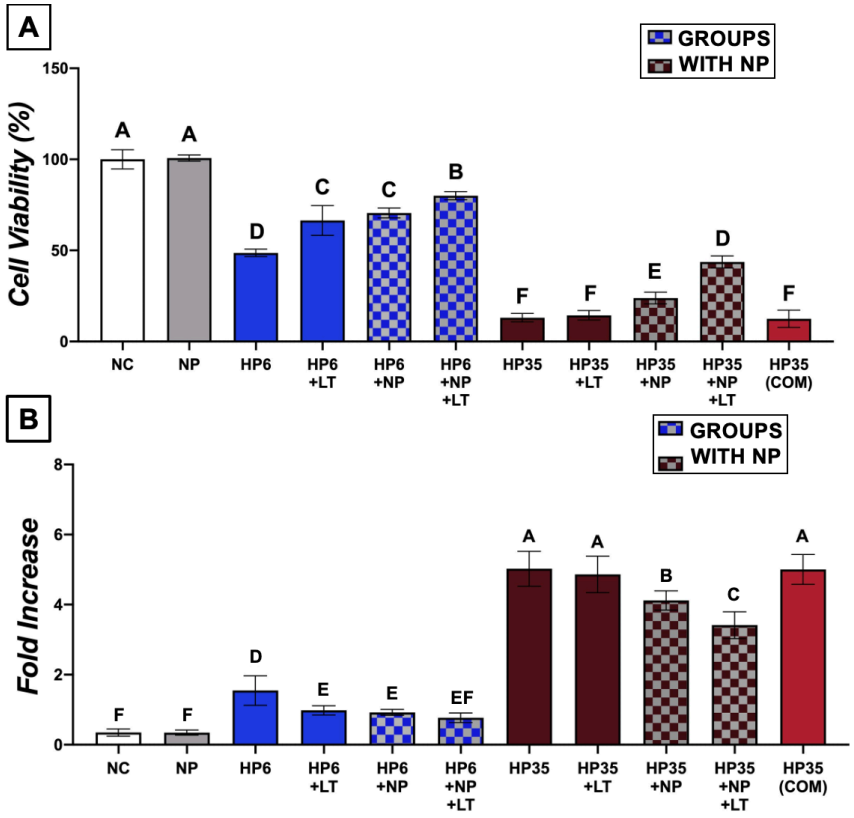

Figure 4. Mean and standard deviation values of cell viability (A) and oxidative stress (B) were calculated at the first bleaching session (T_1). Different letters indicate statistical differences, according to one-way ANOVA and Tukey's test.

Figures 5 and 6 illustrate the cell morphology under SEM following the bleaching protocols. In NC and NP groups, a number of MDPC-23 cells with wide cytoplasm covered almost the entire glass substrate. In HP6, a small number of cells adhered to the substrate was observed in comparison with NC and NP groups. However, higher number of MDPC-23 remained on the glass substrate when NP was incorporated to the gel, which was submitted to LT. In HP35 (experimental and commercial), only a few round-shaped and contracted cells remained attached to the glass in comparison to NC and NP groups. The number of cells exposed to the extracts from experimental HP35 gels seems higher in groups containing NP and irradiated with LT, exhibiting a pattern similar to the HP6 group.

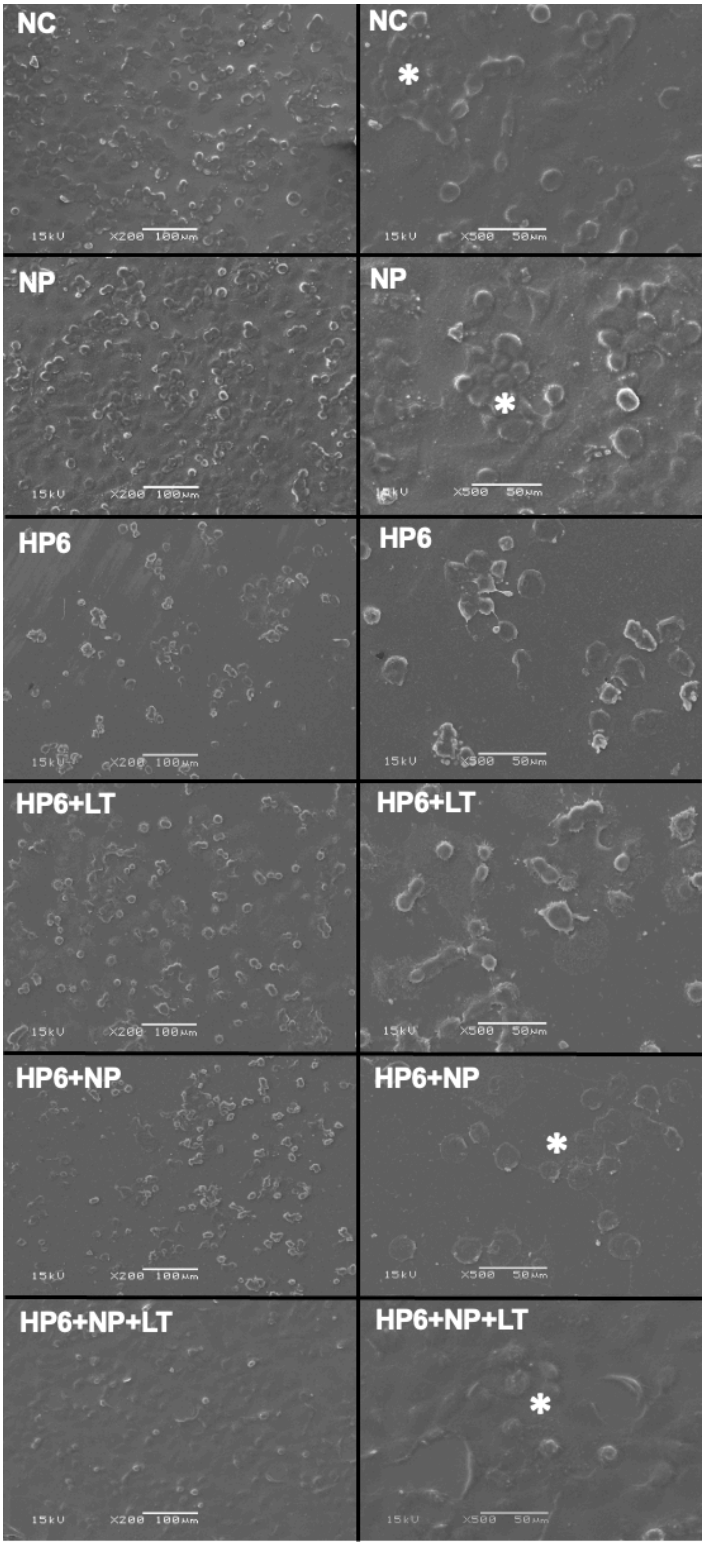

Figure 5. Representative SEM images (200x and 500x) of glass slides on which MDPC-23 cells were cultured and then exposed to extracts collected from the APCs after the bleaching protocols. In NC and NP groups, a high number of round-shaped cells remained attached to the glass. Some of those cells were in mitosis (*). The number of cells with morphology similar to NC and NP was remarkably reduced in HP6 group. Note that the number of MDPC-23 cells in HP6+LT, HP6+NP, and HP6+NP+LT was higher than in HP6 group.

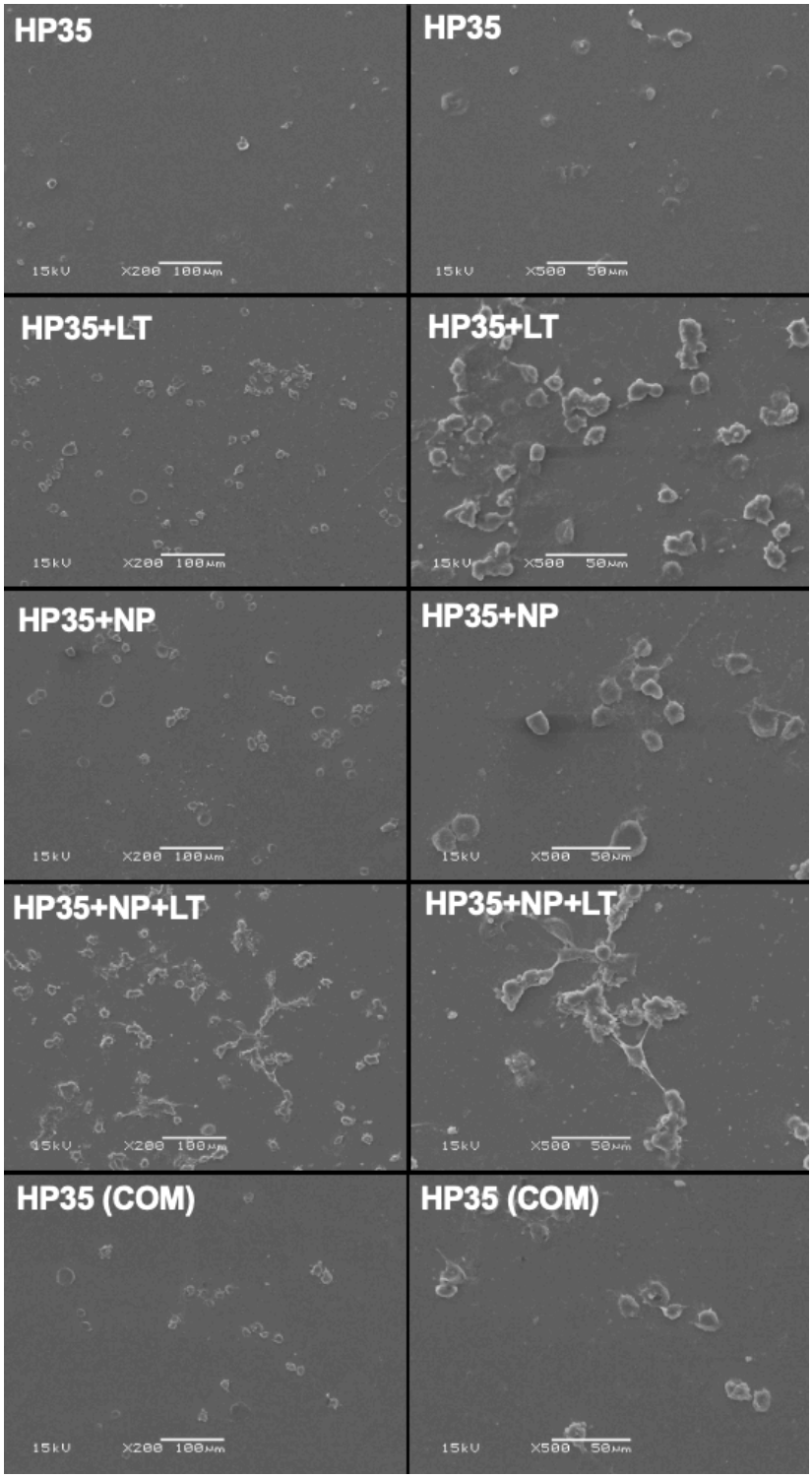

Figure 6. Representative SEM images (200x and 500x) of glass slides on which MDPC-23 cells were cultured and then exposed to extracts collected from the APCs after the bleaching protocols. In HP35 and HP35 (COM), most of lethally damaged cells detached from the glass substrate, on which only fragments of death cells were seen. The number of cells with morphology similar to NC and NP groups was higher in HP35+LT, HP35+NP, and HP35+NP+LT than in HP35 and HP35 (COM).

Figure 7 displays the fluorescence microscopy images of the Live/Dead assay. NC and NP exhibited a higher number of viable cells stained with Calcein AM (green color) than observed in commercial and all the experimental HP35 groups. In HP35 (COM), most of cells with disrupted cytoplasm membrane were stained with EthD-1 (red). HP35+NP+LT presents slightly more live cells and a lower number of red staining than the other experimental HP35 groups. All the HP6 groups exhibited a high number of viable cells, such as observed in NC and NP.

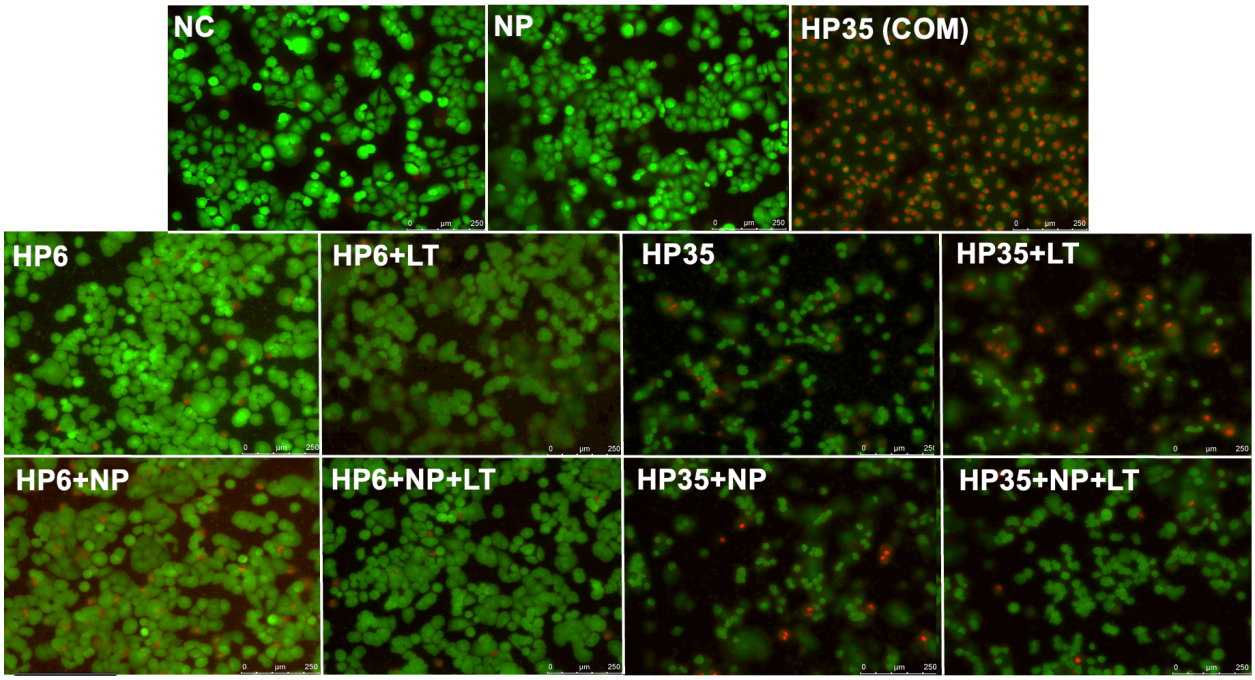

Figure 7. Representative images of live (green) and dead (red) cells exposed to the extracts collected after the bleaching protocols. NC and NP showed that viable cells were entirely stained with Calcein AM (green). HP35 groups presented a larger number of cells with disrupted cytoplasm membrane (stained with EthD-1 probe – red color) in comparison to all other groups assessed. The incorporation of NP and irradiation with LT seem to have increased the number of live cells and slightly decreased the red staining in the HP35+NP+LT.

DISCUSSION

Violet light irradiation or the incorporation of co-doped TiO_2 nanoparticles increased the mean ΔE_{00} and ΔWI_D values attained by experimental 6% HP gels. However, the combination of nanoparticles and light (HP6+NP+LT) further enhanced the color and whiteness changes, significantly increasing in about 100% the ΔWI_D in comparison to HP6 group. Therefore, the first null hypothesis that the NP incorporation would not increase the efficacy of the experimental gels was rejected. Also, the HP6+NP+LT protocol rendered esthetic outcomes similar to those attained with the light-irradiated HP35 groups even containing only one-fifth of the H_2O_2 concentration. These findings corroborate the first report of these experimental bleaching protocols, in which the incorporation of 5% of the co-doped titanium dioxide nanoparticles not only enhanced the esthetic outcomes of HP6 but also upheld the gel's pH and preserved the enamel mineral content following bleaching [15].

The metaloxide nanoparticles in this study that were synthesized using solvothermal reactions were previously reported to be predominantly in the anatase phase, to have a well-defined pore-size distribution, to be electron deficient, and to generate substantial amounts of ROS [16] even in dark conditions [24], thereby indicating that the nanoparticles investigated could have spontaneously accelerated the H_2O_2 decomposition even. However, it has been well-described in the literature that the fundamental mechanism by which TiO_2 generates ROS depends on light irradiation using appropriate wavelengths (i.e., UV-C, UV-B, or UV-A) [18]. When such an electronic requirement is reached, the electrons from the valence band (fundamental state) are promoted into the conduction band (excited state). As a result, the valence band presents an electron vacancy that is positively charged and likely to recombine with the conduction's free electrons, thereby releasing heat or light [25]. However, if this recombination does not occur, continuous generation of positive holes and excited electrons migrate to the surface of the nanoparticles and may participate in various oxi-reduction reactions in the surface of the photocatalyst, including the generation of longer-lived ROS by follow-on reactions (i.e., hydroperoxyl - $\cdot OOH$) [18].

In this direction, the TiO_2 nanoparticles used in this study were already proven to render an optical absorption spectrum in the violet light range (λ = 390-420 nm) twice as

much as a commercially available TiO_2 nanoparticle (P25, Degussa), which could be explained by the TiO_2 doping with other chemical elements (nitrogen and fluorine) in the present study [26]. Therefore, the light source (λ = 401.82 nm) [19] used and the length of the irradiation protocol (30-min total) might have accelerated H_2O_2 dissociation as well as favored follow-on reactions in the bulk of the TiO_2, generating longer-lived ROS. Supposedly, these ROS would interact more intensely with the long-chained chromophores that are responsible for the discoloration of dentin[4]. Previous studies showed that the violet irradiation by itself was responsible for increasing the esthetic outcomes of low-concentrated bleaching gels [14, 27, 28], but possibly by increasing the gel's temperature and increasing the speed of the decomposition of HP into short-lived ROS. Based on findings of the present study, it becomes clear that NP and LT worked synergistically to improve the efficacy of experimental gels containing 6% of H_2O_2, which highlights the importance of combining light and nanoparticles to render relevant esthetic outcomes as optimal as possible when using a low-concentrated gel for in-office bleaching.

There was a slight increase in the mean ΔE_{00} and ΔWI_D values for HP35+LT and HP35+NP groups, but NP and LT combination did not act synergically in this instance. An expected higher number of free radicals in HP35+NP+LT could have interacted with each other and not with the chromophores [29, 30]. Carlos et al. (2022) also showed that the incorporation of TiO_2 nanotubes into 35% HP gel alongside with violet LED irradiation did not enhance the efficacy of in-office tooth bleaching [31]. Likewise, some reports also pointed out that the violet LED light itself did not significantly improve the ΔE_{00} and ΔWI_D of high-concentrated gels (35-40%) [27, 32]. Thus, it could be speculated that the high concentration of H_2O_2 in a gel is already sufficient to promote ΔE_{00} and ΔWI_D values compatible with excellent tooth bleaching efficacy [33], which would refuse the necessity of approaches to improve the efficacy attained by a 35% HP gel.

On the other hand, the incorporation of NP into experimental HP35 gels decreased the trans-amelodentinal diffusion of H_2O_2 that reduced the oxidative stress and consequently the toxic effects of the bleaching protocol to pulp cells. These effects were positively affected by the violet LED irradiation of the gels. Hence, the second null hypothesis that the co-doped nanoparticles would not increase the cytotoxic effects of the

experimental bleaching gels was accepted. In view of this fact, even though HP35+NP+LT did not render better esthetic outcomes compared to both commercial and experimental HP35 groups, this approach might have indeed accelerated the decomposition of HP into ROS, thereby increasing their interaction with each other, and decreasing the number of non-reacted H_2O_2 diffusing towards the culture medium (extracts). Similarly, Martins et al. (2022) recently demonstrated that the application of a nanofiber scaffold and a heme-peroxidase enzyme-based polymeric primer on the enamel surface also protected the cell viability and minimized the oxidative stress and quantification of H_2O_2 against 35% H_2O_2 hydrogen combined with violet LED [34].

However, it is still possible to observe that all HP35-containing protocols in the present study not only dramatically reduced the cell viability (from ≅ 50% [HP35+NP+LT] to ≅ 90% (HP35 and HP35 [COM]), but also altered the cell morphology, disruptured the cell membranes (EthD-1 red fluorescence signal) and led to the highest levels of oxidative stress. High concentrations of ROS are directly correlated with increases in the oxidative stress due to the accumulation of oxidized-damaged molecules in the cells, impairing the cellular homeostasis [35]. Moreover, ROS can react with lipidic structures from the cell membranes, leading to lipid peroxidation initiation and, consequently, membrane rupture [36]. An *in vivo* study showed that 35% H_2O_2 significantly increased the number of ROS in human pulp, showing a positive relation with the presence of lysosomal cathepsin B enzymes, inferring that the oxidative stress influences the protein matrix degradation and this relates to the inflammatory and death process of the pulp cells [37]. On the other hand, Duque et al. (2017) reported that lower concentration of H_2O_2 resulted in higher expression of odontoblast differentiation predictors (alkaline phosphatase activity and mineralized nodule deposition) in human pulp cells after 14 and 21 days from bleaching with 10% when compared to 35% H_2O_2 [38]. Therefore, cells exposed to lower concentration of H_2O_2 would be more prone to regulate their oxidative stress and regenerative potential over time.

Despite the similar cell viability between HP35+NP+LT and HP6 groups, reducing the H_2O_2 concentration to as low as 6% was noticeably more effective in decreasing the diffusion of H_2O_2 and, more importantly, the oxidative stress. In fact, previous researchers revealed that cellular oxidative stress caused by H_2O_2 works in a concentration-dependent

manner [23]. In the HP6-containing groups, the use of LT and NP separately was already responsible for upregulating the % of MDPC-23 cells viability and downregulating their oxidative stress. Nonetheless, HP6+NP+LT reached the most favorable results, indicating, once more, a higher H_2O_2 decomposition and/or ROS that interacted longer with the staining molecules in dentin. The highest percentage of cell viability following this experimental protocol could be related to the significantly lower trans-amelodentinal H_2O_2 diffusion and the similar oxidative stress to the negative control ($p > 0.05$). Besides, representative SEM images revealed that the cell morphology in HP6+NP+LT maintained the characteristics seen in the NC and NP. Previous studies stated that decreasing the application time and concentration of H_2O_2 in the bleaching gels reduces the cytotoxicity of bleaching protocols, including the preservation of the cell morphology [9, 23]. However, taking into consideration the SEM images and data from other tests performed in the present investigation, one may consider that NP incorporation and LT irradiation would provide an additional protection factor to the cells.

Recently, the incorporation of manganese oxide into 6% and 10% H_2O_2 gels further irradiated with a violet LED light exhibited a similar pattern to HP6+NP+LT in terms of esthetic outcomes and cytotoxic effects [13, 22], which reinforces that bleaching approaches with photocatalyst-containing gels could protect the viability and minimize the negative impacts of bleaching treatments onto cells homeostasis. Even though these *in vitro* data should be carefully extrapolated into the clinical settings, experimental materials containing low concentrations of HP and NP were shown to render esthetic results that were comparable to those attained with 35%- containing gels while significantly reducing the adverse effects on pulp-like cell cultures in a validated artificial pulp chamber model. The results of the present study could also indicate that experimental materials and techniques investigated could possibly decrease dentin hypersensitivity and negative effects on enamel microhardness and chemical makeup.

Despite the inherent limitation of an *in vitro* study, the use of enamel-dentin disks coupled with the APCs is a technique well-established in the literature as an approach to mimic the trans-amelodentinal diffusion of H_2O_2 [9, 23, 38, 39]. When reaching the pulp tissue, odontoblasts underlying the dentin would be the first cells exposed to highly toxic components. In this sense, the immortalized odontoblast-like

MDPC-23 cell line with a similar phenotype to human odontoblast was used [40]. However, it is important to bear in mind that the vital human pulp holds other organic structures, and it receives exudation pressure from the dentinal fluid, which is likely to influence the internal diffusion of H_2O_2. Therefore, future *in vitro* and *in vivo* studies are paramount to confirm these data and to further investigate the clinical efficacy and adverse effects of co-doped TiO_2 nanoparticles incorporation into bleaching gels.

CONCLUSIONS

The present findings demonstrated that the increase in the efficacy of the experimental 6% hydrogen peroxide gel was positively and synergically affected by the incorporation of titanium dioxide nanoparticles co-doped with nitrogen and fluorine when subjected to violet LED irradiation. Independently of the peroxide concentration considered, the incorporation of the nanoparticles into the experimental bleaching gels significantly decreased the cytotoxic effects of bleaching protocols, and this scenario was positively influenced by light irradiation. Within all the in-office bleaching treatments, light-irradiated 6% hydrogen peroxide gel incorporated with nanoparticles rendered the most positive maintenance of odontoblast-like cells with a similar esthetic outcome when compared to a commercial 35% hydrogen peroxide bleaching gel.

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3. DISCUSSÃO

Os resultados obtidos no *Artigo 1* confirmaram que o comprimento de onda do LED utilizado encontra-se, de fato, na faixa de luz visível violeta (λ = 401.82 nm). Este mesmo comprimento de onda apresentou alta taxa de absorção pelo esmalte dental (1 mm), aproximadamente 98%, o que confirma o efeito estritamente superficial da luz violeta. Estudos anteriores especularam que este LED é compatível com o pico de absorção de pigmentos extrínsecos (Zanin et al., 2016, Rastelli et al., 2018, Kury et al., 2020b), fato que foi confirmado pela análise de eficácia, a qual demonstrou maior alteração de cor e das coordenadas euclidianas em amostras pigmentadas artificialmente com chá preto e fumaça de tabaco. Previamente, Kobayashi et al., (2021) reportou não haver diferença na alteração de cor e índice de clareamento causados por LED frente aos diferentes protocolos de pigmentação de dentes utilizados (café, vinho tinto e tabaco). Contudo, os presentes achados indicariam que a composição do pigmento depositado na superfície do esmalte dental pode influenciar nesta interação. Por exemplo, o tabaco pode apresentar traços de cádmio, elemento que absorve energia fotônica na faixa de luz violeta e azul (Callister 2018). Sendo assim, o tratamento apenas com LED violeta, poderia ser indicado a pacientes tabagistas. Todavia, estudos clínicos randomizados devem confirmar esta premissa.

Levando em consideração que os voluntários incluídos no ensaio clínico randomizado e controlado (*Artigo 2)* relataram não serem fumantes e que todos foram submetidos à profilaxia dental previamente à avaliação colorimétrica e ao clareamento dental, torna-se justificável o fato do LED violeta ter provocado alterações de cor e índice de clareamento menos evidentes que em estudos *in vitro* (Gallinari et al., 2019, Kury et al., 2020b, Kobayashi et al., 2021) Além disso, a instabilidade de irradiância e heterogeneidade do feixe de luz dependentes do local da ponteira e distância do aparelho à superfície (*Artigo 1)*, poderia ter um impacto direto nos resultados clínicos, uma vez que estas características influenciaram diretamente na energia fotônica entregue ao longo de toda a ponteira do aparelho. Neste contexto, o aperfeiçoamento de LEDs violeta com tecnologias mais avançadas quanto à dissipação de calor ou a incorporação de um filtro acoplado à ponteira poderiam favorecer estes quesitos. Entretanto, a distância do aparelho aos arcos dentais, bem como o tempo de irradiação, ainda merece serem

considerados e estudados, uma vez que há relato *in vitro* de aumento na temperatura pulpar de aproximadamente 8ºC mesmo quando a ponteira é posicionada à distância de 8 mm no protocolo de duração total de 30 minutos (Guanaes et al., 2022).

O acompanhamento clínico revelou que, após 6 e 12 meses, houve aumento significativo de ΔE_{00} para 35% HP irradiado com LED, em comparação a 35% HP sem luz. Contudo, este aumento significativo, comparando LED/CP a CP, foi detectado apenas após 12 meses. Em outras palavras, a irradiação teria favorecido a manutenção do efeito clareador de CP após 1 ano. Numericamente, CP (sem LED) foi o único grupo que apresentou queda para ambos ΔE_{ab} e ΔE_{00} entre T_{6m} e T_{12m}. Deve-se considerar que, apesar da alta concentração de CP (37%) empregada neste estudo, este gel ainda apresenta apenas 1/3 de sua concentração total em peróxido de hidrogênio e que a ureia presente na sua composição pode retardar a decomposição de CP em HP. Tendo isto em vista, o aumento da temperatura do gel em decorrência da irradiação violeta (Costa et al., 2022b) poderia ter acelerado a decomposição química neste gel, aumentando a quantidade de radicais livres (de curta duração) que interagiram com os cromóforos dentinários e prolongando a longevidade deste tratamento (LED/CP). É importante ressaltar que não houve diferenças significativas entre LED/CP e HP sem LED ao longo do estudo, o que aliado a menores índices de sensibilidade reportado durante o tratamento com LED/CP (Kury et al., 2020c), seria uma alternativa possível para pacientes com histórico de hipersensibilidade.

Todavia, não foi detectado aumento significativo para o ΔWI_D de LED/CP e LED/HP comparado a CP e HP sem luz, respectivamente, em T_{6m} e T_{12m}. Este índice (*Whitening Index for Dentistry*) determina a mudança da superfície dental ou de materiais dentários para um maior nível de branco quando sua alteração é positiva (Luo et al., 2009), o que o torna extremamente relevante para análise de eficácia do clareamento dental. Considerando os limiares de percepção de ΔWI_D previamente estabelecidos (Pérez et al., 2018), o tratamento com altas concentrações de HP ainda foi mais perceptível que LED/CP, mesmo não havendo diferença significativa entre eles. Vale ressaltar, porém, que mesmo que os protocolos com HP tenham exibido ΔWI_D superiores após 6 e 12 meses, maior intensidade de sensibilidade foi reportada pelos pacientes durante os tratamentos (Kury et al., 2020c).

Neste contexto, a incorporação de nanopartículas de TiO_2 co-dopadas com nitrogênio e flúor *(Artigo 3)* em géis experimentais de HP em baixas concentrações (6% e 15%) poderia superar a performance de géis de CP mediante a irradiação com LED. De fato, a incorporação favoreceu principalmente a eficácia alcançada por 6% HP e irradiado com LED violeta *in vitro*, o que corrobora outros estudos com outras partículas fotocatalisadoras (Martins et al., 2022, Dias et al., 2022). Independentemente dos resultados obtidos com as demais concentrações de HP, o fato de o gel com sua menor concentração ter superado as médias de ΔE_{00} e ΔWI_D obtidas por 35% HP, seria um indicativo de redução de efeitos adversos causados pela terapia clareadora convencional.

O mecanismo fundamental do dióxido de titânio depende de sua interação com um comprimento de onda de luz apropriado (ou seja, 385 nm para anatase) (Foster et al., 2011). Quando isto ocorre, os elétrons da camada de valência (estado fundamental) são promovidos para a camada condutora (estado excitado). Como resultado, a vacância eletrônica na camada de valência é carregada positivamente e provavelmente se recombina com os elétrons livres da condução, liberando calor ou luz (Miguel Pelaez et al., 2012). No entanto, se essa combinação não ocorrer, a geração contínua de elétrons positivos e negativos pode ocorrer e participar de várias reações de oxi-redução na superfície do fotocatalisador, incluindo a geração de ROS de vida mais longa por reações subsequentes (por exemplo, hidroperoxil - .OOH) (Foster et al., 2011).

Sendo assim, o protocolo de irradiação testados nos *Artigos 1 e 2* podem ter favorecido a ação da nanopartícula de TiO_2. Estudos prévios já haviam incorporado nanopartículas comerciais de TiO_2 dopadas ou não apenas com nitrogênio, mas os resultados não foram promissores (Martin et al., 2015, Bortolatto et al., 2016). Uma das possíveis explicações seria o comprimento de onda azul da geração anterior de LEDs para clareamento, uma vez que o espectro de absorção óptica do TiO_2 é mais próximo da região ultravioleta quando funcionalizado com outros elementos (Esteban Florez et al., 2018). Ademais, a rota de síntese optada para síntese das presentes nanopartículas co-dopadas pode ter positivamente influenciado os achados, já que o método solvo-térmico resultou na obtenção de partículas esféricas com tamanho de 6 a 10 nm, elétron-deficientes e predominantemente na fase anatase, que é a mais estável do dióxido de titânio (Esteban Florez et al., 2020).

Apesar da maior manutenção dos picos de carbonato e fosfato presentes no esmalte dental após clareamento com géis incorporados com as nanopartículas, todos os protocolos clareadores experimentais acarretaram diminuição significativa da proporção mineral após avaliação por meio de FT-IR (*Artigo 3*). Porém, mediante as mesmas condições e independente do LED violeta, géis contendo TiO_2 preveniram a redução significativa da microdureza de superfície do esmalte e aumentaram os valores médios de KHN em profundidade (*Artigo 4)*, o que poderia estar ligado à funcionalização de flúor ao TiO_2 (Pitts et al., 2017), mas também às características inerentes ao espessante de escolha (carbopol). Naturalmente, o carbômero apresenta um pH acídico e as nanopartículas, por sua vez, aumentaram levemente este pH (*Artigo 3*). Ressalta-se, contudo, que o cálcio solubilizado do esmalte dental poderia complexar com os ânions do polímero em gel, tornando o mesmo subsaturado em relação à superfície (van der Reijden et al., 1997). Portanto, a incorporação das nanopartículas em um biopolímero ou polímeros com pH neutros ou alcalinos poderia revelar se o benefício das nanopartículas à preservação da microdureza do esmalte clareado é independente do tipo de espessante.

Embora imagens representativas de microscopias de força atômica (*Artigo 3*) e eletrônica de varredura (*Artigo 4*) tenham revelado alterações pontuais em alguns grupos, a análise quantitativa de *Ra* (*Artigo 4*) revelou não haver influência significativa de nenhum dos protocolos experimentais testados sobre a rugosidade de superfície. Estes achados também são importantes do ponto de vista da microbiologia oral, uma vez que com altos valores médios de Ra acumulariam mais biofilme (Ittatirut et al., 2014), devido ao aumento da área e energia de superfície. Sendo assim, esperava-se que a manutenção de rugosidade notada ao longo do estudo não acarretaria diferenças na atividade metabólica de *S Mutans* aderidos à superfície do esmalte clareado. Contudo, alguns grupos, inclusive com nanopartículas, apresentam atividade metabólica bacteriana significativamente maior ou menor que o controle negativo, mas não foram utilizados os mesmos espécimes nos *Artigos 3 e 4*. Deve-se levar em consideração, ainda, que a capacidade antimicrobiana do TiO_2 (Barão et al., 2022) poderia promover algum efeito latente na superfície do esmalte clareado e que não há relatos na literatura sobre esta possível modulação do esmalte dental clareado. Segundo os achados do

Artigo 3, todavia, todos os grupos clareados apresentaram atividade bacteriana metabólica mediante um ensaio de bioluminescência, ou seja, a incorporação de nanopartículas não foi capaz de impedir a formação de biofilme em superfícies clareadas.

Finalmente, os dados obtidos no *Artigo 5* revelaram que tanto a incorporação das nanopartículas como a irradiação do LED violeta diminuíram significativamente os efeitos citotóxicos do clareamento em consultório. Neste estudo, novamente foi observado que as estratégias experimentais foram capazes de manter a eficácia do clareamento dental mesmo utilizando concentração cinco vezes menor de HP. Outros compostos fotocatalisadores, como o óxido de manganês, têm demonstrado comportamento semelhante, aumentando a eficácia do clareamento com 6 a 10% HP e reduzindo significativamente a difusão de peróxido, morte celular, estresse oxidativo e alterações na morfologia de células de linhagem odontoblásticas (Dias et al., 2022, Ribeiro et al., 2022b, Ribeiro et al., 2022c).

Interessantemente, o uso de LED em 6% HP promoveu melhora significativa na porcentagem de viabilidade celular e níveis de estresse oxidativo. Isto demonstraria, como discutido no *Artigo 2* que, de fato, a irradiação com esta fonte luminosa pode aumentar a decomposição de HP em ROS (curta duração) e, por conseguinte, diminuir a difusão de HP na câmara pulpar. Entretanto, o protocolo experimental utilizando 6% HP com 5% de nanopartículas e irradiados com LED violeta acarretou níveis de manutenção celular mais próximos, ou até sem diferença significativa, do grupo controle, ao passo que se aproximou mais dos resultados estéticos alcançados por 35% HP *(Artigos 3 e 5)*. A possível explicação para melhores resultados obtidos para o grupo 6%HP+5%TiO_2+LED em comparação a 6%HP+LED poderia residir na geração de ROS com meia-vida mais longa, devido ao mecanismo de ação de TiO_2 discutido anteriormente, levando a uma interação mais longa com os cromóforos dentinários antes destes serem consumidos.

Espera-se, assim, uma manutenção mais segura da estrutura pulpar de dentes clareados e, consequentemente, menor sensibilidade pós-operatória e transitória em decorrência da aplicação de um gel com 6% HP e incorporado com 5% NP e irradiado com LED violeta. Mesmo frente às presentes evidências de que o uso deste gel experimental e da irradiação luminosa poderiam promover um clareamento eficaz e

seguro à superfície e subsuperfície do esmalte, bem como à polpa dental, ensaios clínicos randomizados e controlados ainda são essenciais para a indicação clínica deste protocolo clareador. Estes ensaios clínicos devem, sobretudo, acompanhar a longevidade dos protocolos propostas, sendo que após 12 meses de acompanhamento pode haver recidiva de cor para algum dos protocolos e utilizar índices atualizados de avaliação colorimétrica, visto que o ΔWI_D comportou-se de maneira diferente de ΔE_{00}, dentro de um mesmo tempo de avaliação. Não menos importante, a utilização de um LED violeta com irradiância e feixes de luz mais estáveis pode ser primordial para a garantia da sua ação sob as nanopartículas de dióxido de titânio co-dopadas.

4. CONCLUSÃO

- O aparelho LED violeta (Bright Max Whitening, MMOptics) apresenta feixe de luz heterogêneo e irradiância instável em função do tempo de irradiação, região e posição de sua ponteira, levando a maior eficácia in vitro, por si só, mediante à presença de pigmentos extrínsecos provenientes da fumaça de cigarro.
- Clinicamente, o LED violeta sozinho não promoveu eficácia clareadora satisfatória e aumentou significativamente a alteração de cor, mas não o índice de clareamento, promovida pelos géis comerciais de peróxidos de carbamida 37% e de hidrogênio 35% em uma avaliação após 12 meses. A irradiação de 37% CP não apresentou diferença significativa em comparação a 35% HP para nenhum dos índices avaliados, mesmo após 1 ano.
- A incorporação de nanopartículas de dióxido de titânio co-dopadas com nitrogênio e flúor em géis clareadores experimentais elevou o pH e a eficácia obtida pelos géis de menores concentrações, sendo que agiu sinergicamente com LED violeta nos resultados obtidos por 6% HP ao atingir os níveis de eficácia de 35% HP. Porém, os géis experimentais contendo as nanopartículas não foram capazes de impedir a formação de biofilme de *S Mutans* na superfície do esmalte clareado.
- Embora a aplicação de todos os géis experimentais tenha reduzido a proporção mineral entre carbonato e fosfato do esmalte dental, o clareamento com os géis contendo as nanopartículas não influenciou negativamente na microdureza, rugosidade e morfologia de superfície, bem como na microdureza transversal e proporção entre cálcio e fósforo do esmalte dental.
- Contudo, os efeitos citotóxicos em decorrência do clareamento com 35% HP foram significativamente reduzidos à medida que houve redução da concentração de HP, incorporação de nanopartículas e irradiação com LED violeta. A irradiação do gel clareador de 6% HP com nanopartículas acarretou maior viabilidade celular e menores níveis de difusão de peróxido e estresse oxidativo.

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ANEXO 1 - CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "Análise in vivo do LED violeta na alteração de cor, sensibilidade e conteúdo mineral do clareamento dental", CAAE 72879717.7.0000.5418, dos pesquisadores Vanessa Cavalli Gobbo, Marcelo Giannini, Maria Carolina Guilherme Erhardt, Daylana Pacheco da Silva, Erika Eiko Waida e Matheus Kury Rodrigues, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 25/09/2017.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "In vivo Purple LED analysis on the color alteration, tooth sensitivity and mineral content of dental bleaching", CAAE 72879717.7.0000.5418, of the researcher's Vanessa Cavalli Gobbo, Marcelo Giannini, Maria Carolina Guilherme Erhardt, Daylana Pacheco da Silva, Erika Eiko Waida and Matheus Kury Rodrigues, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on September, 25 2017.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
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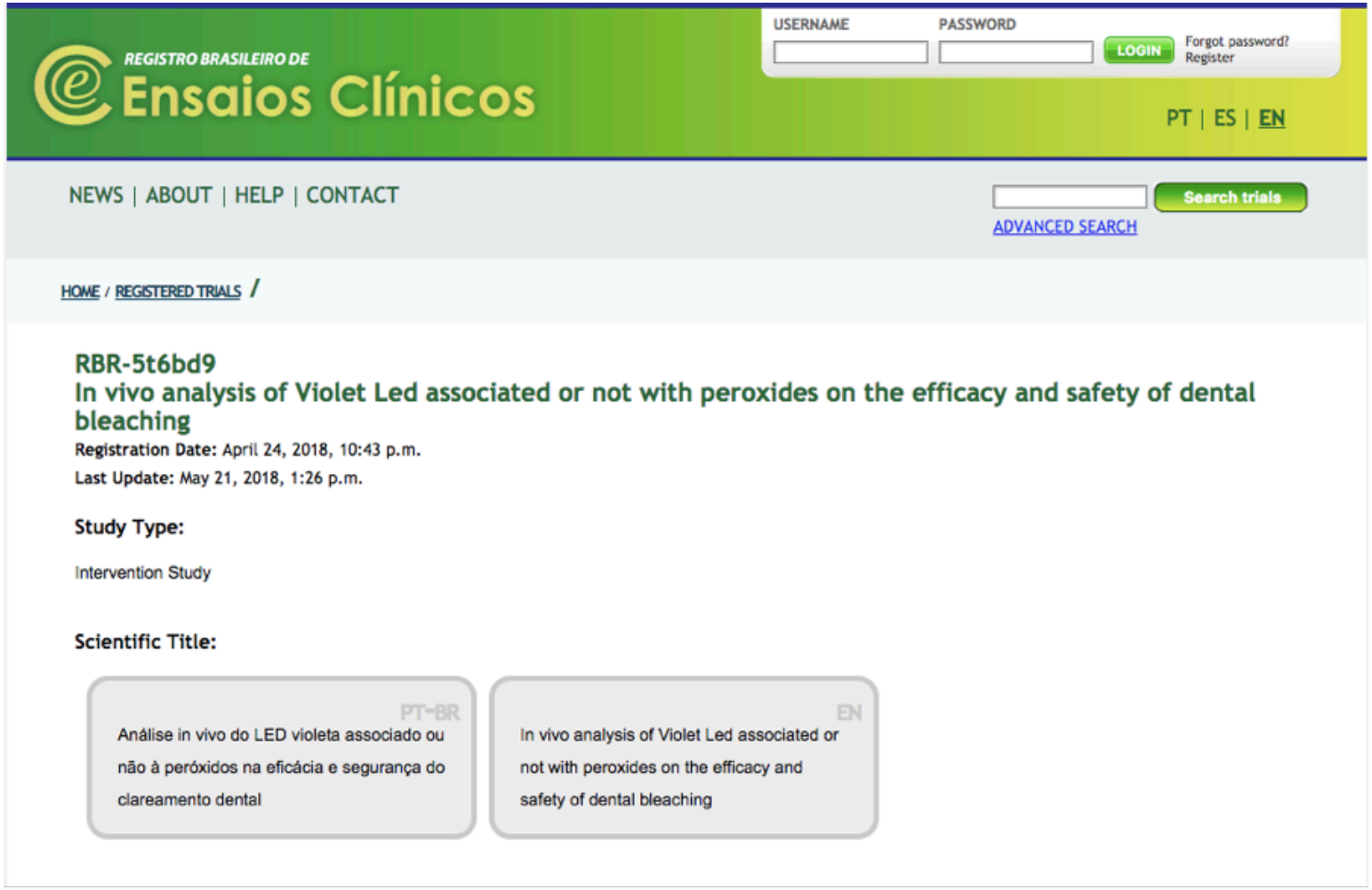

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RBR-5t6bd9

In vivo analysis of Violet Led associated or not with peroxides on the efficacy and safety of dental bleaching

Registration Date: April 24, 2018, 10:43 p.m.

Last Update: May 21, 2018, 1:26 p.m.

Study Type:

Intervention Study

Scientific Title:

PT-BR

Análise in vivo do LED violeta associado ou não à peróxidos na eficácia e segurança do clareamento dental

EN

In vivo analysis of Violet Led associated or not with peroxides on the efficacy and safety of dental bleaching

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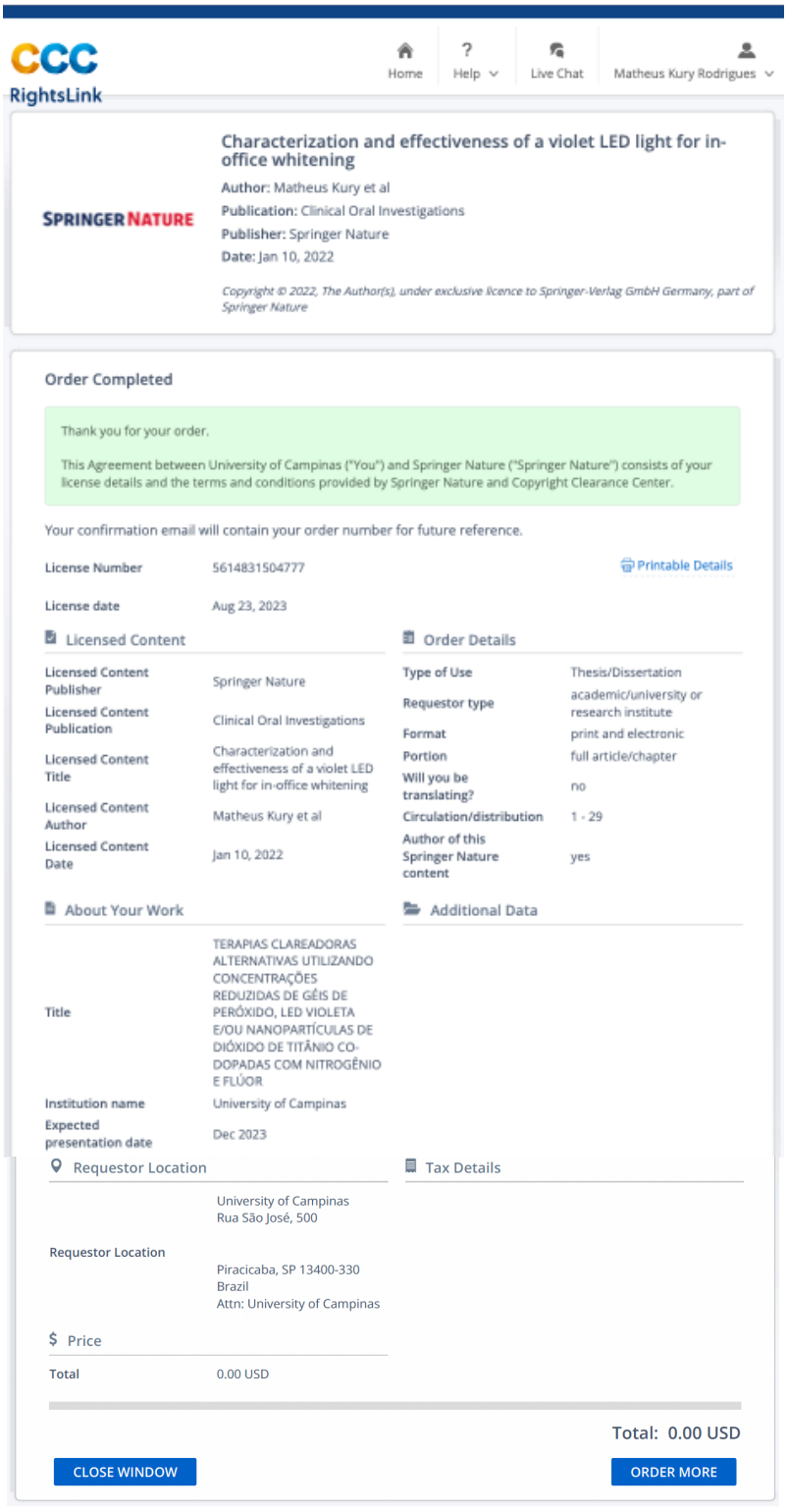
ANEXO 4 – AUTORIZAÇÃO SPRINGER NATURE ARTIGO 1



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SPRINGER NATURE

Characterization and effectiveness of a violet LED light for in-office whitening

Author: Matheus Kury et al
Publication: Clinical Oral Investigations
Publisher: Springer Nature
Date: Jan 10, 2022

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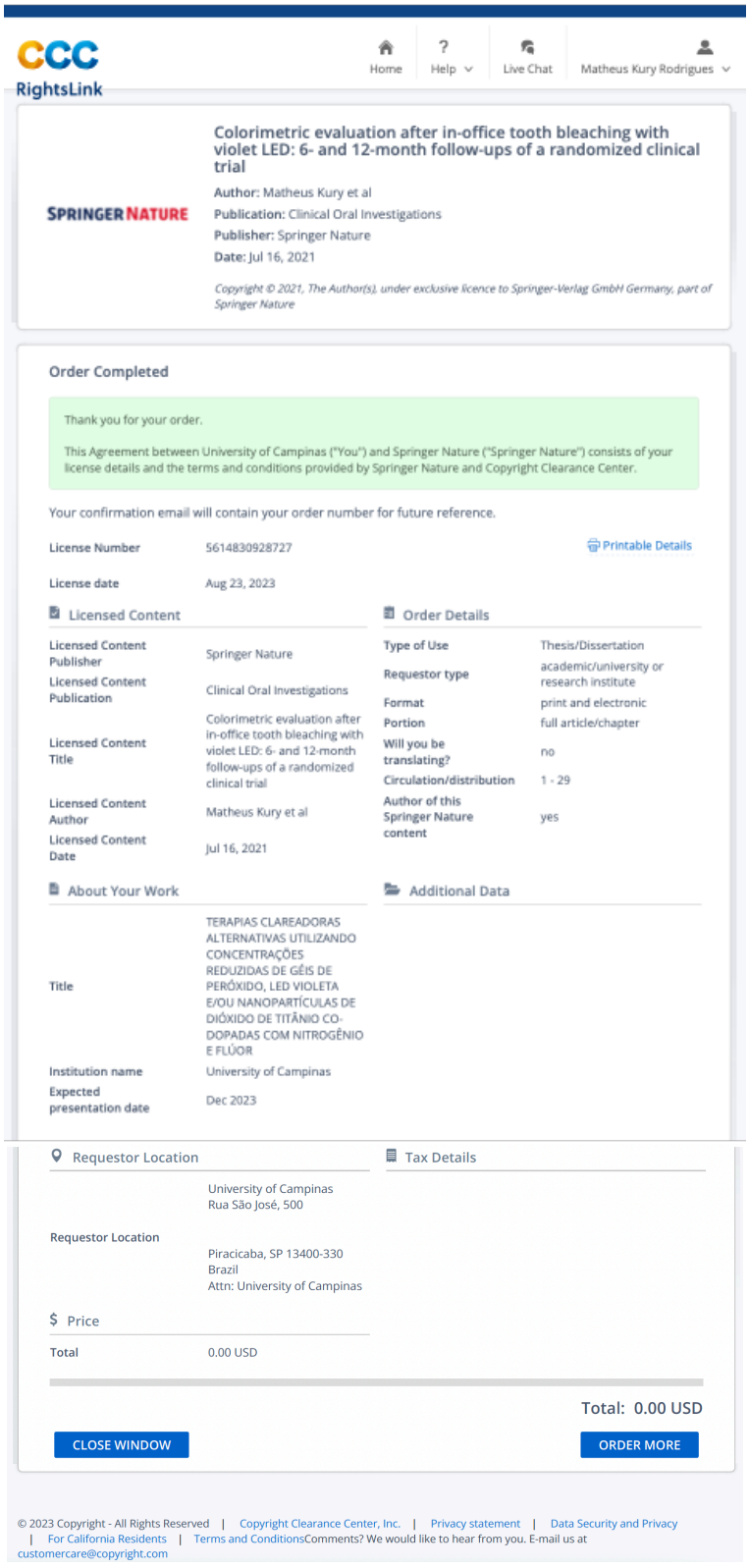

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SPRINGER NATURE

Colorimetric evaluation after in-office tooth bleaching with violet LED: 6- and 12-month follow-ups of a randomized clinical trial

Author: Matheus Kury et al
Publication: Clinical Oral Investigations
Publisher: Springer Nature
Date: Jul 16, 2021

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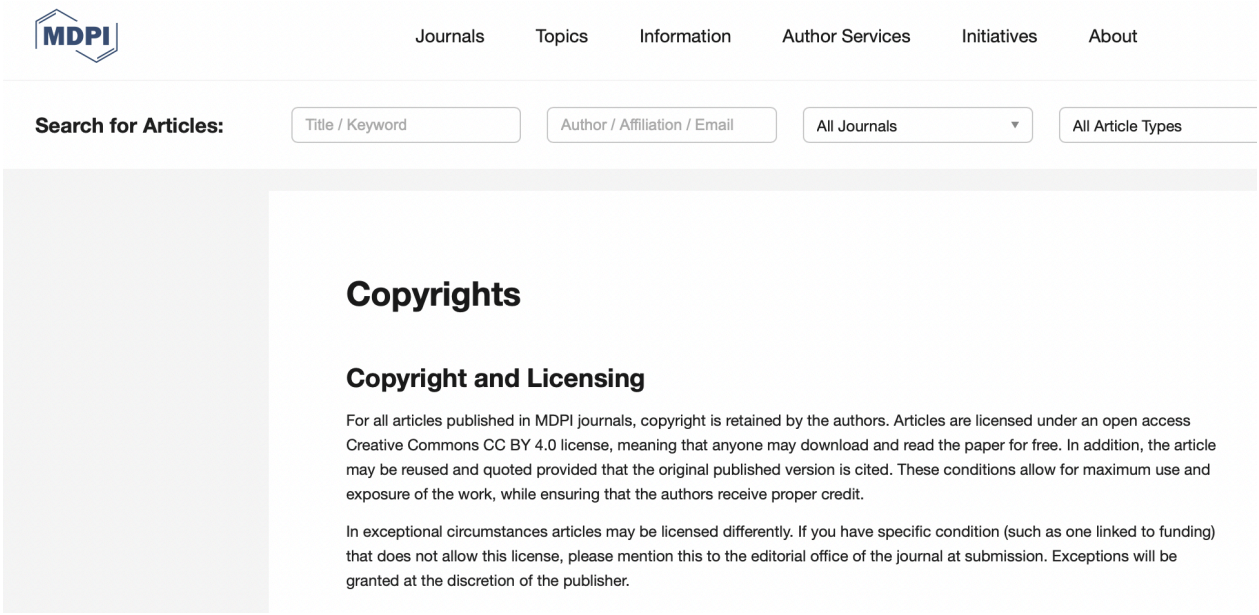

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ANEXO 7 – COMPROVANTE SUBMISSÃO ARTIGO 4

Thursday, December 7, 2023 at 11:30:21 Brasilia Standard Time

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Date: Sunday, November 12, 2023 at 1:48:53 PM Brasilia Standard Time
From: em.jjod.0.8754eb.d12a0912@editorialmanager.com on behalf of Journal of Dentistry
To: Matheus Kury

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Journal: Journal of Dentistry
Title: Effects of experimental in-office bleaching gels incorporated with co-doped titanium dioxide nanoparticles on dental enamel physical properties
Corresponding Author: Dr. Vanessa Cavalli
Co-Authors: Matheus Kury, D.D.S., M.S., Ph.D.; Fernando Luís Esteban Florez, D.D.S., M.S., Ph.D.; Cínthia Machado Pereira Tabchoury, Pharm.D., M.S., Ph.D.
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Journal of Dentistry

ANEXO 8 – COMPROVANTE SUBMISSÃO ARTIGO 5

Wednesday, August 23, 2023 at 11:38:44 Brasilia Standard Time

Subject: Confirm co-authorship of submission to Dental Materials
Date: Tuesday, July 4, 2023 at 9:17:40 AM Brasilia Standard Time
From: em.dentma.0.847337.439afdc3@editorialmanager.com on behalf of Dental Materials
To: Matheus Kury

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Journal: Dental Materials
Title: Co-doped titanium dioxide nanoparticles decrease the cytotoxicity of experimental hydrogen peroxide gels for in-office tooth bleaching
Corresponding Author: Dr. Vanessa Cavalli
Co-Authors: Matheus Kury, D.D.S., M.S.; Rafael Antonio de Oliveira Ribeiro, D.D.S., M.S.; Carlos Alberto Souza-Costa, D.D.S., M.S., Ph.D.; Fernando Luís Esteban Florez, D.D.S., M.S., Ph.D.
Manuscript Number:

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If you did not co-author this submission, please contact the Corresponding Author directly at cavalli@unicamp.br

Thank you,

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