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**NANOGEL TERMORRESPONSIVO CARREGADO COM IBUPROFENO
APLICADO COMO AGENTE ANTI-INFLAMATÓRIO NO CLAREAMENTO
DENTAL: UM ESTUDO *IN VIVO***

SAMARA KELLY SILVA CHAVES FERNANDES MOURA

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Co-orientador: Prof. Dr. Arnóbio Antônio da Silva Júnior

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“Os sonhos não determinam o lugar onde vocês vão chegar, mas produzem a força necessária para tirá-los do lugar em que vocês estão. Sonhem com as estrelas para que vocês possam pisar pelo menos na Lua. Sonhem com a Lua para que vocês possam pisar pelo menos nos altos montes. Sonhem com os altos montes para que vocês possam ter dignidade quando atravessarem os vales das perdas e das frustrações. Bons alunos aprendem a matemática numérica, alunos fascinantes vão além, aprendem a matemática da emoção, que não tem conta exata e que rompe a regra da lógica. Nessa matemática você só aprende a multiplicar quando aprende a dividir, só consegue ganhar quando aprende a perder, só consegue receber, quando aprende a se doar.”

Augusto Cury

RESUMO

A capacidade *in vivo* do nanogel termorresponsivo carregado com ibuprofeno de bioadesão à superfície de dentes submetidos ao clareamento, bem como a liberação do fármaco através dos túbulos dentinários até atingir o tecido pulpar, foram avaliadas em ratos Wistar. Nanoemulsões auto-associadas do tipo óleo em água carregadas com fármaco apresentaram tamanho de gotícula < 150 nm e potencial zeta negativo. As nanoemulsões contendo poloxamer 407 na fase aquosa exibiram tamanho de gotícula reduzido, estabilidade física melhorada, viscosidade termorresponsiva controlada e liberação lenta do fármaco. Diferentes grupos de animais tratados incluíram um grupo controle (CTRL); animais clareados (BLE) (37.5% H₂O₂, Pola Office Plus®, SDI); animais clareados tratados com hidrogel convencional carregado com ibuprofeno (BLE+IBF-GEL), e animais clareados tratados com nanogel termorresponsivo carregado com ibuprofeno (BLE+IBF-NEG). Em comparação com o grupo BLE+IBF-GEL, as imagens de microscopia eletrônica de varredura (MEV) e de microscopia confocal de varredura a laser (MCVL) mostraram a capacidade superior do grupo BLE+IBF-NEG em oferecer uma barreira física na superfície do dente, oclusão dos poros e transporte do corante fluorescente rodamina B para a polpa dentária. A análise morfológica revelou os menores escores de infiltrado inflamatório e ausência de necrose para o grupo BLE+IBF-NEG após 24 horas e 14 dias. Estudos imuno-histoquímicos reforçaram esta hipótese. Os dados experimentais apresentados neste estudo confirmam o incrível potencial de aplicação tópica de um nanogel inovador, de fácil aplicação, utilizado como material inteligente de ação analgésica e antiinflamatória no clareamento dental. O caráter multidisciplinar do estudo possibilitou o seu desenvolvimento e aprimoramento dos resultados obtidos.

Palavras-chave: Nanotecnologia; dispersões coloidais auto-associadas; nanoemulsões; clareamento dental; sensibilidade dentinária; inflamação.

ABSTRACT

The *in vivo* ability of thermosensitive nanogel containing ibuprofen for bioadhesion on the surface of teeth submitted to bleaching and drug delivery through dentinal tubules to the dental pulp were evaluated in Wistar rats. Drug-loaded self-assembled oil in water nanoemulsions presented droplet size < 150 nm and negative zeta potential. Nanoemulsions containing poloxamer 407 in the aqueous phase exhibited reduced droplet size, improved physical stability, controlled the thermosensitive viscosity, and slow drug release. Different treated animals' groups included a control group (CTRL); bleached animals (BLE) (37.5% H₂O₂, Pola Office Plus®, SDI); bleached animals treated with a conventional ibuprofen-loaded hydrogel (BLE+IBF-GEL), and bleached animals treated with thermosensitive ibuprofen-loaded nanogel (BLE+IBF-NEG). Compared to the BLE+IBF-GEL group, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) images showed the superior ability of BLE+IBF-NEG group to offer a physical barrier on the tooth surface, pores occlusion, and transport the fluorescent dye rhodamine B to dental pulp. Morphological analysis revealed the lowest inflammatory infiltrate scores and absence of necrosis for BLE+IBF-NEG group after 24 hours and 14 days. Immunohistochemical studies reinforced this hypothesis. The experimental data presented in this study confirm the incredible potential for topical and easy application of a novel and smart nanogel, as analgesic and anti-inflammatory treatment for bleaching procedure. The multidisciplinary nature of the study enabled its development and improvement of the results obtained.

Keywords: Nanotechnology; self-assembled colloidal dispersions; nanoemulsions; tooth bleaching; dentin sensitivity; inflammation.

LISTA DE ABREVIATURAS E SIGLAS

ARRIVE (Animal Research: Reporting of *In Vivo* Experiments)

B-GEL: Hidrogel branco

BLE: Grupo Clareado

BLE+IBF-GEL: Grupo clareado tratado com hidrogel convencional carregado com ibuprofeno

BLE+IBF-NEG: Grupo clareado tratado com nanogel termorresponsivo carregado com ibuprofeno

B-NE: Nanoemulsão branca

B-NEG: Nanogel branco

CEUA: Comissão de Ética no Uso de Animais

COX – 1: Ciclooxygenase 1

COX – 2: Ciclooxygenase 2

CTRL: Grupo Controle

EDTA: Ethylenediamine Tetraacetic Acid

g: Grama

HPMC: Hidroxipropilmetilcelulose

H₂O₂: Peróxido de hidrogênio

IBF: Ibuprofeno

IBF-GEL: Hidrogel convencional carregado com ibuprofeno

IBF-NE: Nanoemulsão carregada com ibuprofeno

IBF-NEG: Nanogel termorresponsivo carregado com ibuprofeno

IL-10: Interleucina-10

IL-1 β : Interleucina-1 beta

kg: Quilo

LAICI: Laboratório de Investigação de Câncer e Inflamação

MCVL: Microscopia confocal de varredura a laser

MEV: Microscopia eletrônica de varredura

MF: Microscopia de fluorescência

mg: Miligrama

mL: Mililitro

mm: Milímetro

MS: Mistura de surfactante

NE: Nanoemulsão

NEG: Nanogel

nm: Nanômetro

O/A: Óleo em água

PBS: Solução salina tamponada com fosfato

Pdl: Índice de polidispersão

pH : Potencial hidrogeniônico

PO80: Polissorbato 80

PZ: Potencial Zeta

p/p: Peso/peso

P407: Poloxamer 407

RPM: Rotações por minuto

SP: Substância P

SPC: Fosfatidilcolina de soja

UV: Ultravioleta

µm: Micrómetro

κ: Kappa ponderado

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1. INTRODUÇÃO

A alteração de cor dos dentes apresenta etiologia variável que vai desde causas extrínsecas, relacionadas à adsorção de substâncias na superfície do esmalte¹, a intrínsecas, onde a cor é associada às propriedades de absorção e reflexão de luz da dentina e do esmalte², como as observadas na hipoplasia, no manchamento por tetraciclina e na amelogenese e dentinogenese imperfeitas³. Além disso, o próprio processo do envelhecimento, no qual ocorre maior formação de dentina secundária, leva à observação de dentes mais escurecidos⁴.

Assim, cada vez mais pacientes buscam por soluções estéticas nos consultórios odontológicos, a fim de resolver as alterações de cor dentárias para obter dentes mais claros, estando em evidência o clareamento dental⁵, o qual vem atraindo cada vez mais investimentos do mercado odontológico. Este procedimento é um tratamento estético eficaz e conservador quando bem indicado⁵⁻⁷, entretanto continua sendo um desafio em termos de controle da sensibilidade, a qual é relatada principalmente com a utilização do peróxido de hidrogênio (H₂O₂), em concentrações em torno de 35%, utilizado no clareamento de consultório⁸⁻¹⁰. Esta sintomatologia faz com que alguns pacientes optem por não realizar o tratamento ou até mesmo descontinuem o protocolo clareador, sendo uma de suas causas de insucesso¹¹.

O H₂O₂ e seus produtos de degradação, as espécies reativas de oxigênio, apresentam um alto potencial de penetração no dente através dos túbulos dentinários devido ao seu baixo peso molecular^{12,13}. O processo de clareamento é decorrente da oxidação da matriz orgânica do esmalte¹⁴ e possivelmente pela oxidação dos aminoácidos aromáticos presentes na fosfoproteína dentinária, responsável pelas propriedades de fluorescência da dentina¹⁵.

Deste modo, as alterações estruturais no esmalte e dentina^{16,17}, assim como as respostas inflamatórias observadas no tecido pulpar¹⁸⁻²² promovidas pelo clareamento estão relacionadas diretamente à concentração e ao tempo de exposição ao peróxido, bem como ao número de sessões clareadoras²³, sendo a intensidade da resposta pulpar inversamente relacionada à espessura do esmalte²⁴.

O clareamento dental afeta diretamente os componentes orgânicos dos tecidos dentários e promove mudanças na fase mineral que resultam em alterações morfológicas visíveis em superfície²⁵⁻²⁷, sendo observados aumento da porosidade e a exposição dos processos de Tomes que são sugestivos do aumento da sensibilidade²⁸. Imagens de microscopia eletrônica de varredura permitiram observar em estudos *in vitro* áreas com diferentes níveis de desmineralização do esmalte,

remoção parcial da camada aprismática, exposição dos prismas e perda de substância interprismática promovidas pelo H₂O₂ a 35%²⁸⁻³⁰. Além disso, redução significativa da dureza e do módulo de elasticidade do esmalte foram observados, bem como aumento de trincas e diminuição da resistência à fratura³¹.

A penetração do peróxido de hidrogênio através dos prismas de esmalte até atingir a câmara pulpar pode ser observada ao se adicionar a este a rodamina B³². Esta é uma molécula pouco solúvel em água e de baixo peso molecular frequentemente utilizada como um agente fluorescente para determinar a direção do fluxo, e quando aplicada na superfície externa do dente apresenta capacidade de penetração na estrutura dentária³³. Portanto, através da microscopia confocal a laser pode-se identificar o sinal de substâncias fluorescentes^{32,33} e de tecidos³⁴, sendo obtidas imagens com alta qualidade^{35,36}.

Quanto às alterações observadas no tecido pulpar submetido ao clareamento é observado que este apresenta diferentes intensidades de infiltrado inflamatório, elevada migração de macrófagos, degradação do colágeno, aumento da vasodilatação, mudanças visíveis na camada odontoblástica, além de zonas de necrose por liquefação e coagulação^{18,20,23,24,37}. Logo, a avaliação das alterações morfológicas e de mediadores inflamatórios utilizados para mapear as alterações pulpares após a exposição ao agente clareador é fundamental para elucidar como esta relação se estabelece. Foi observado um aumento na expressão de citocinas pró-inflamatórias, como a ciclooxigenase 2 (COX-2) e a interleucina 1 beta (IL-1 β), na polpa de grupos submetidos ao clareamento em relação ao controle^{38,39}, bem como do neuropeptídeo, substância P (SP)^{37,40}. Níveis mais elevados de bradicinina, bem como da citocina anti-inflamatória interleucina-10 (IL-10), foram identificados em tecido pulpar humano diagnosticado com pulpite irreversível^{41,42}. Entretanto, a expressão destas substâncias relacionadas ao clareamento dental ainda é desconhecida.

Nesse sentido, a fim de obter alternativas capazes de prevenir a sensibilidade causada pelo clareamento, alguns estudos em humanos mostraram que a administração sistêmica de ibuprofeno (IBF) foi capaz de reduzir os sintomas durante⁴³ ou mesmo até a primeira hora após o tratamento⁴⁴. Além disso, estudos recentes em animais relataram resultados positivos para o uso de substâncias com ação anti-inflamatória aplicadas topicamente no esmalte após o tratamento clareador, como o otosporin e o carvedilol, minimizando os danos pulpares promovidos por este procedimento^{20,37,45}.

O ibuprofeno (ácido (RS)-2-(4-(2-metilpropil) fenil) propanóico) é um inibidor não seletivo das ciclooxigenases (COX-1 e COX-2), enzimas envolvidas na síntese de prostaglandinas. É um fármaco cristalino e não termolábil^{46,47}, com baixa solubilidade em água (0,02 - 0,09 mg / mL)⁴⁸, o que limita a biodisponibilidade oral. Sua rápida degradação no estômago e o considerável metabolismo de primeira passagem são desafios adicionais para o desenvolvimento de uma fórmula farmacêutica tópica alternativa e eficiente⁴⁹.

Em vista disso, alternativas nanotecnológicas, como as nanoemulsões (NEs), têm demonstrado potencial superior para penetração tópica quando comparadas aos sistemas convencionais⁵⁰. Entre os nanocarreadores utilizados para encapsular drogas pouco solúveis em água, as NEs são sistemas coloidais cineticamente estáveis nas quais líquidos imiscíveis são misturados como gotículas estabilizadas por surfactantes. As NEs do tipo óleo em água (O / A) são capazes de encapsular drogas hidrofóbicas, como o IBF, em gotículas de óleo (100 a 600 nm) dispersas em água⁵¹⁻⁵³. Estes nanocarreadores podem penetrar em canais micrométricos como os túbulos dentinários e transportar o fármaco para o tecido alvo, controlando a sua liberação e aumentando sua eficácia^{54,55}. Apesar dessas vantagens, as NEs apresentam baixa viscosidade, fato que limita seu uso para aplicação tópica na odontologia. Esta limitação pode ser contornada com o uso de hidrogéis na fase aquosa, melhorando a bioadesão⁵⁶. Portanto, a utilização de um copolímero termorresponsivo, como o poloxamer 407, parece ser uma alternativa promissora para o aumento *in situ* da viscosidade da NE e formação de um nanogel (NEG) na superfície dentária, melhorando a bioadesão *in vivo* da dispersão coloidal. Este NEG termorresponsivo oferece ganhos práticos incríveis quando comparado a dispersões líquidas e a hidrogéis convencionais⁵⁷.

O presente estudo objetiva desenvolver uma formulação inovadora de um nanogel termorresponsivo carregado com IBF capaz de ser utilizado como uma abordagem analgésica e anti-inflamatória tópica no clareamento dental e avaliar *in vivo* sua bioadesão na superfície dentária, sua capacidade de penetração através dos túbulos dentinários e de transporte do fármaco para polpa de molares de ratos Wistar. Uma avaliação criteriosa das composições capazes de produzir o nanogel termorresponsivo auto-associado carregado com IBF é realizada. Hipótese nula: o nanogel carregado com ibuprofeno não é capaz de reduzir a inflamação no tecido pulpar quando aplicado antes e após o clareamento dental.

2. JUSTIFICATIVA

Estudos *in vivo* são importantes para avaliar interações a nível celular, bem como investigar níveis seguros de toxicidade e eficácia dos fármacos. Assim, observou-se que poucos estudos *in vivo* foram desenvolvidos a fim de elucidar os efeitos do peróxido de hidrogênio, bem como a sua associação a terapias anti-inflamatórias, sobre o tecido pulpar.

Deste modo, a partir da observação na literatura dos resultados promissores do ibuprofeno administrado sistemicamente na redução da sensibilidade promovida pelo clareamento, bem como dos resultados positivos da administração tópica de medicamentos na redução da inflamação pulpar gerada pelo peróxido, este estudo experimental justifica-se pela necessidade de redução dos danos pulpares gerados por este procedimento a partir do desenvolvimento de uma nanoformulação inovadora carregada com ibuprofeno de aplicação tópica que apresente *in vivo* potencial de bioadesão e penetração no elemento dentário.

Portanto, esta abordagem inédita além de possibilitar a redução dos efeitos colaterais decorrentes da administração sistêmica de fármacos, apresenta-se como uma opção terapêutica alternativa e promissora que permita no futuro a redução da sensibilidade relatada pelos pacientes submetidos ao tratamento clareador.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar o potencial anti-inflamatório do nanogel de ibuprofeno sobre as alterações pulpareas promovidas pelo clareamento dental em ratos Wistar.

3.2 Objetivos específicos

Desenvolver e caracterizar a formulação do nanogel termorresponsivo auto-associado carregado com ibuprofeno;

Avaliar *in vivo* o potencial de bioadesão do nanogel de ibuprofeno sobre o esmalte dentário de ratos Wistar submetidos ao clareamento;

Avaliar *in vivo* a capacidade de penetração do nanogel de ibuprofeno no elemento dentário;

Analisar as alterações morfológicas e imuno-histoquímicas do tecido pulpar submetido ao clareamento e à administração tópica do nanogel de ibuprofeno.

4. MÉTODO

4.1. Materiais

O ibuprofeno foi adquirido da All Chemistry (Jabaquara, Brasil). O triglicerídeo de cadeia média (Miglyol 812®) foi adquirido da Sasol (Hamburgo, Alemanha), a fosfatidilcolina de soja (95%) (SPC) foi obtida da Avanti Polar Lipids (Alabama, EUA), o polissorbato 80 (PO80; Tween 80®) e o poloxamer 407 (P407; Pluronic F127®) foram adquiridos da Sigma-Aldrich (St. Louis, MO, EUA), e o propilenoglicol foi adquirido da Qhemis (São Paulo, Brasil). Água purificada foi obtida através do sistema de osmose reversa (modelo OS50 LX, Gehaka Ins. Ltd, Brasil). Os demais reagentes e produtos foram de grau analítico.

4.2. Preparação do nanogel termorresponsivo carregado com IBF

4.2.1 Teste de solubilidade do ibuprofeno

A solubilidade do IBF foi testada em diferentes ingredientes utilizados nas formulações (Miglyol 812®, fosfatidilcolina de soja, Tween 80® e propilenoglicol). Um excesso do fármaco foi misturado com 2 mL de cada matéria-prima, sob agitação magnética constante (360 rpm) por 48 horas a 25°C, adaptado de Dukovski et al.⁵⁸ As dispersões foram centrifugadas por 5 minutos a 5.000 rpm. O sobrenadante (1 mL) foi diluído em 10mL em frascos volumétricos com etanol⁵⁹. A absorbância foi mensurada ($\lambda = 264$ nm) e o percentual de fármaco foi calculado utilizando a equação ($y = 0,0017x - 0,0143$; $r^2 = 0,9999$) obtida a partir da construção da curva padrão, sob as mesmas condições analíticas, considerando parâmetros de validação analítica⁶⁰.

4.2.2. Preparação das nanoemulsões

As nanoemulsões foram preparadas pelo método de inversão de fase⁵³. A água purificada foi lentamente gotejada (1 mL/mL) na fase oleosa contendo uma mistura de óleo de soja, mistura de surfactante (MS; SPC:PO80, 3:1 p/p) e co-solvente propilenoglicol, a 65°C, sob agitação magnética a 1.500 rpm durante 40 minutos. A temperatura de 65°C foi utilizada considerando o ponto de fusão do fármaco (cerca de 80°C) e a estabilidade térmica neste intervalo^{46,47}. As formulações básicas consistiram em 1% (p/p) de óleo de soja e 10% (p/p) de propilenoglicol. A MS nas formulações variou de 1% (p/p) a 5% (p/p) e água de 88% (p/p) a 84% (p/p), respectivamente. O mesmo método foi utilizado para as NEs carregadas com IBF, em que 1% (10 mg/mL) do fármaco foi dissolvido na fase oleosa. Após a preparação, as NEs foram filtradas e a carga do fármaco foi medida nas NEs, conforme descrito

para o teste de solubilidade do IBF. A composição detalhada das NEs é descrita na Tabela 1.

Tabela 1. Composição das diferentes formulações.

Formulações	Composição (% p/p)							
	IBF	Óleo	SPC	PO80	Co-solvente	P407	HPMC	Água
B-NE10	0.0	1.0	0.75	0.25	10.0	0.0	0.0	88.0
B-NE25	0.0	1.0	1.875	0.625	10.0	0.0	0.0	85.5
B-NE50	0.0	1.0	3.75	1.25	10.0	0.0	0.0	84.0
IBF-NE10	1.0	1.0	0.75	0.25	10.0	0.0	0.0	87.0
IBF-NE25	1.0	1.0	1.875	0.625	10.0	0.0	0.0	84.5
IBF-NE50	1.0	1.0	3.75	1.25	10.0	0.0	0.0	83.0
B-NEG100	0.0	0.0	0.75	0.25	10.0	10.0	0.0	78.0
B-NEG125	0.0	0.0	0.75	0.25	10.0	12.5	0.0	75.5
B-NEG150	0.0	0.0	0.75	0.25	10.0	15.0	0.0	73.0
B-NEG175	0.0	0.0	0.75	0.25	10.0	17.5	0.0	70.5
B-NEG200	0.0	0.0	0.75	0.25	10.0	20.0	0.0	68.0
IBF-NEG100	1.0	1.0	0.75	0.25	10.0	10.0	0.0	77.0
IBF-NEG125	1.0	1.0	0.75	0.25	10.0	12.5	0.0	74.5
IBF-NEG150	1.0	1.0	0.75	0.25	10.0	15.0	0.0	72.0
IBF-NEG175	1.0	1.0	0.75	0.25	10.0	17.5	0.0	69.5
IBF-NEG200	1.0	1.0	0.75	0.25	10.0	20.0	0.0	67.0
B-GEL	0.0	0.0	0.0	0.0	0.0	0.0	2.0	98.0
IBF-GEL	1.0	0.0	0.0	0.0	0.0	0.0	2.0	97.0

Nota: Ibuprofeno (IBF), fosfatidilcolina de soja (SPC), polissorbato 80 (PO80), poloxamer 407 (P407), hidroxipropilmetilcelulose (HPMC), nanoemulsão branca (B-NE), nanoemulsão carregada com IBF (IBF-NE), nanogel branco (B-NEG), nanogel carregado com IBF (IBF-NEG), hidrogel branco (B-GEL), hidrogel carregado com IBF (IBF-GEL).

4.2.3. Preparação dos nanogéis

Os nanogéis termorresponsivos foram preparados dissolvendo P407 (Pluronic F127® nas NEs contendo 1% de MS; 24 horas após a preparação das NEs) como agente gelificante a 4°C sob homogeneização em vortex por 2 minutos. Diferentes concentrações de P407 foram testadas (10% p/p, 12,5% p/p, 15% p/p, 17,5% p/p e 20% p/p). Para comparações de desempenho *in vivo*, um hidrogel convencional carregado com ibuprofeno foi preparado na ausência da NE utilizando 2% de hidroxipropilmetilcelulose (HPMC) em água. A composição detalhada dos NEGs é descrita na Tabela 1.

4.2.4. Tamanho da gotícula e potencial zeta das nanoemulsões

O tamanho médio das gotículas e o índice de polidispersão (Pdl) das formulações foram avaliados por dispersão de luz dinâmica utilizando o equipamento

Malvern Zetasizer Nano ZS (Malvern Ins. Ltd, UK). As amostras foram diluídas 1:100 em água ultrapura e analisadas a 25°C em um ângulo de detecção de 173° e comprimento de onda de 633 nm. Os valores obtidos são expressos em média \pm desvio padrão (n = 2). Os potenciais zeta foram medidos com o mesmo equipamento, sob as mesmas condições, avaliando a mobilidade eletroforética. As amostras também foram diluídas 1: 100 em água ultrapura.

4.2.5. Estabilidade física das nanoemulsões

Antes dos experimentos de doseamento do fármaco, a estabilidade física das formulações brancas foi monitorada durante 60 dias a 25°C. As NEs carregadas com a droga foram monitoradas por 15 dias sob as mesmas condições. As amostras foram armazenadas em frascos de vidro hermeticamente fechados. O tamanho médio das gotículas, Pdl e potencial zeta foram medidos conforme descrito no item 4.2.4.

4.2.6. Doseamento do fármaco encapsulado

A quantidade de 0,5 g das formulações foi dissolvida em 2 mL de metanol e completada em 10 mL com etanol em frascos volumétricos⁶¹. As soluções filtradas foram analisadas em espectrofotômetro UV-Vis a 264 nm, conforme descrito para o teste de solubilidade do IBF.

4.2.7. Estudo de liberação *in vitro*

A liberação *in vitro* da droga foi estudada utilizando células de difusão vertical de Franz a 37°C⁶². O compartimento receptor foi preenchido com solução salina tamponada com fosfato (PBS; pH = 7,4; 11 mL \pm 1) mantida sob agitação constante (100 rpm) e separada do compartimento doador por uma membrana de acetato de 0,45 μ m (previamente hidratada em PBS por 24 horas). Em intervalos de tempo específicos, alíquotas de 1 mL foram removidas para análise do fármaco por espectrofotometria UV-Vis a 264 nm. O mesmo volume de PBS foi repostado ao meio imediatamente após para manter a condição sink.

4.3. Estudos *in vivo*

4.3.1. Animais

Todos os procedimentos experimentais seguiram as diretrizes ARRIVE (Animal Research: Reporting of *In Vivo* Experiments)⁶³ para pesquisa animal sugerida pelo Centro Nacional para Redução, Refinamento e Substituição no uso de animais em pesquisa. Todos os protocolos animais foram aprovados pelo Comitê de Ética em Pesquisa Institucional (CEUA 037/2016) da Universidade Federal do Rio Grande do Norte (UFRN, Rio Grande do Norte, Brasil). Os animais foram fornecidos

pelo Biotério do Centro de Ciências da Saúde/UFRN, alojados em condições-padrão (5 animais por gaiola, feita de polipropileno natural; temperatura controlada de $22 \pm 1^\circ\text{C}$ e 70% de umidade) em ciclos de 12 horas de claro / escuro com acesso *ad libitum* a água e ração para roedores (*Presence*®) (Figura 1).



Figura 1 – Armazenamento dos animais em gaiola.
Fonte: a autora.

Quarenta e oito ratos machos (*Rattus norvegicus*) pesando entre 250 e 300 gramas foram utilizados neste estudo de boca-dividida. Destes, 30 animais foram utilizados para análises morfológicas e imuno-histoquímicas. Os animais foram distribuídos aleatoriamente em quatro grupos (5 animais/grupo): grupo CTRL, grupo BLE, grupo BLE+IBF-GEL e grupo BLE+IBF-NEG, e avaliados após 24 horas e 14 dias. Os molares superiores e inferiores foram utilizados no estudo boca-dividida e em todos os grupos. Os molares esquerdos foram utilizados nos grupos experimentais, enquanto os molares direitos foram usados como grupo controle, totalizando $n = 10$ hemiarcadas analisadas por grupo. O nanogel termorresponsivo carregado com IBF selecionado para os estudos *in vivo* foi a formulação IBF-NEG150 (Tabela 2; Figura 2). Todos os experimentos *in vivo* utilizaram formulações recém-preparadas (intervalo de 24 horas) e as amostras carregadas com fármaco tiveram o conteúdo analítico da droga determinado.

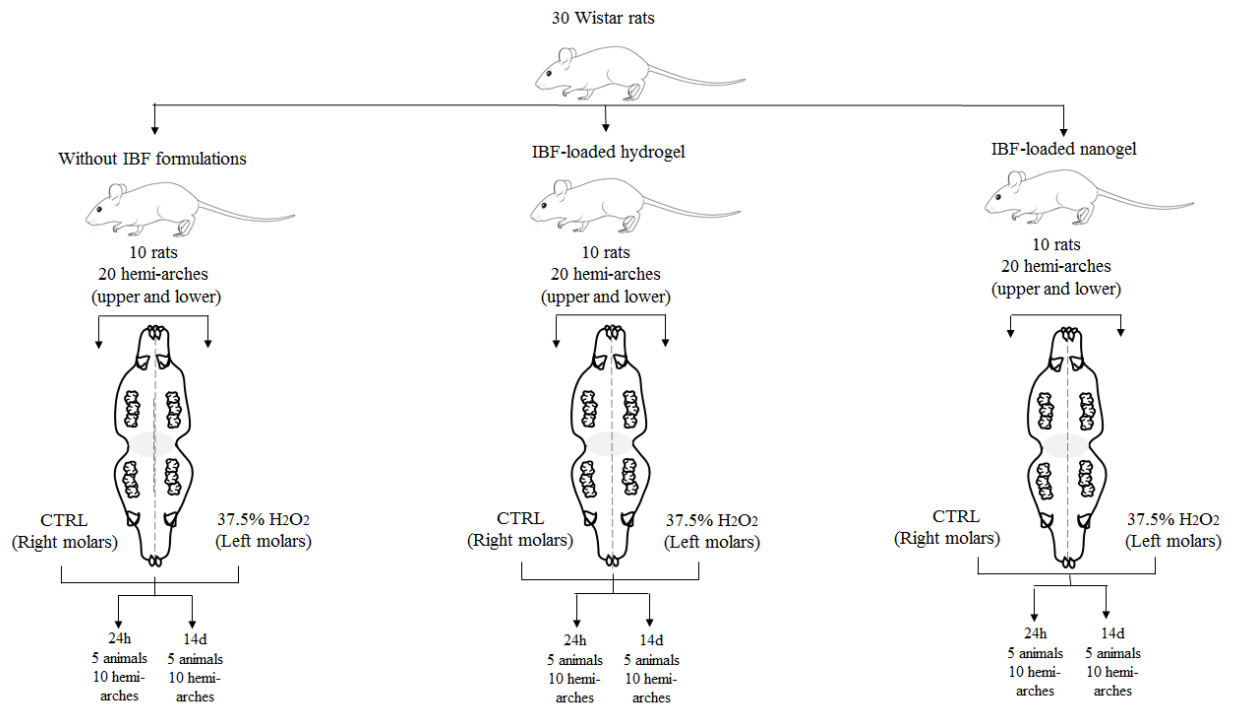


Figura 2. Fluxograma de alocação dos animais aos grupos de estudo.

Tabela 2. Grupos de tratamento e protocolos de aplicação.

Grupo	Descrição	Substâncias	Protocolo de Aplicação	Período de avaliação 24 horas	Período de avaliação 14 dias
CTRL	controle	Nenhuma	Nenhuma	5 animais* (n=10 hemiarcadas)	5 animais* (n=10 hemiarcadas)
BLE	clareados	37,5% H ₂ O ₂ (Pola Office Plus®, SDI)	3 aplicações (8 minutos cada)	5 animais (n=10 hemiarcadas)	5 animais (n=10 hemiarcadas)
BLE+IBF-GEL	Animais clareados tratados com hidrogel convencional carregado com IBF	37,5% H ₂ O ₂ IBF-GEL (Composição especificada na Tabela 1)	IBF-GEL+ 37,5% H ₂ O ₂ ** + IBF-GEL	5 animais (n=10 hemiarcadas)	5 animais (n=10 hemiarcadas)
BLE+IBF-NEG	Animais clareados tratados com nanogel termorresponsivo carregado com IBF	37,5% H ₂ O ₂ IBF-NEG150 (Composição especificada na Tabela 1)	IBF-NEG150 + 37,5% H ₂ O ₂ ** + IBF-NEG150	5 animais (n=10 hemiarcadas)	5 animais (n=10 hemiarcadas)

* Os animais do grupo controle foram escolhidos aleatoriamente entre os animais dos grupos experimentais no estudo boca-dividida.

** O agente clareador foi aplicado a todos os grupos seguindo o mesmo protocolo descrito para o grupo 2.

Quatro grupos de cinco animais em cada grupo, totalizando 10 hemiarcadas analisadas por grupo.

Experimentos iniciais foram realizados em quatro animais (grupo BLE+B-NEG150). Destes, dois animais foram analisados após 24 horas, e dois animais foram analisados após 14 dias, seguidamente à aplicação do nanogel branco (B-NEG150) nos molares superiores e inferiores esquerdos, totalizando oito hemiarcadas avaliadas. A metodologia de aplicação para o grupo BLE+IBF-NEG foi seguida para analisar possível interferência da presença do IBF no desempenho da substância em análise. O pequeno número de animais não permitiu a realização de análises estatísticas (Material suplementar).

Os demais animais foram divididos aleatoriamente para análises qualitativas por microscopia eletrônica de varredura (MEV), microscopia de fluorescência e microscopia confocal de varredura a laser (MCVL). Os protocolos foram realizados sob anestesia intraperitoneal com cloridrato de cetamina 10% (Syntec, Brasil; 70 mg/kg) e cloridrato de xilazina 2% (Syntec, Brasil; 7 mg/kg) (Figura 3). Todos os

animais foram pesados previamente ao início dos procedimentos e à eutanásia (Figura 4). Para realização dos procedimentos, os animais foram imobilizados em uma mesa de trabalho preconizada por Erasquim e Muruzábal⁶⁴ e modificada por Carvalho⁶⁵ (Figura 5).



Figura 3 – Anestesia intraperitoneal.
Fonte: a autora.



Figura 4 – Pesagem dos animais
Fonte: a autora



Figura 5 – Mesa de trabalho para realização dos procedimentos *in vivo*.
Fonte: a autora.

4.3.2. Clareamento dental e protocolos anti-inflamatórios

Após a profilaxia (Figura 6), todos os grupos experimentais receberam aplicações do agente clareador H_2O_2 a 37,5% (Pola Office Plus®, SDI, Austrália) (Figura 7) de acordo com as recomendações do fabricante (Tabela 2), e os tecidos periodontais foram protegidos com uma barreira gengival resinosa (Top Dam, FGM, Brasil). Os animais dos grupos BLE+IBF-GEL e BLE+IBF-NEG (Tabela 2) receberam aplicações das respectivas formulações carregadas com IBF (Figura 8) 10 minutos antes da aplicação do agente clareador e 10 minutos após sua remoção, adaptado de Gallinari et al.³⁷. As substâncias foram aplicadas com auxílio de microbrush, com volume aproximado de 0,01 mL por hemiarcada (Figura 9). Todos os produtos aplicados nos dentes foram removidos com o auxílio de uma cânula de aspiração (Figura 10) e os dentes lavados com água. Os procedimentos foram realizados por uma dentista.

Após a realização dos protocolos, os animais foram observados em sala de controle pós-operatório do Núcleo de Cirurgia Experimental/UFRN e eutanasiados de acordo com os períodos de avaliação. A eutanásia foi realizada com overdose intraperitoneal de tiopental sódico (100 mg/kg). Posteriormente, as hemiarcadas

foram removidas (Figura 11) e armazenadas em solução fixadora de formol tamponado a 10% por 24 horas para as análises morfológicas e imunohistoquímicas.



Figura 6 – Profilaxia dos elementos dentários.

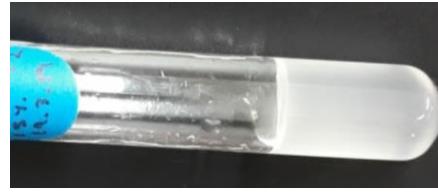
Fonte: a autora.



Figura 7 - Peróxido de hidrogênio a 37,5% (Pola Office Plus®, SDI, Austrália).
Fonte: a autora.



(A)



(B)

Figura 8 – Hidrogel convencional de ibuprofeno (A) aplicado no grupo BLE+IBF-GEL, e Nanogel termorresponsivo de Ibuprofeno (B) aplicado no grupo BLE+IBF-NEG. As substâncias foram confeccionadas no Laboratório de Tecnologia Farmacêutica e Biotecnologia/Departamento de Farmácia/UFRN.

Fonte: a autora.



Figura 9 – Aplicação das formulações nos elementos dentários.

Fonte: a autora.



Figura 10 - Remoção das formulações com o auxílio de uma cânula de aspiração.
Fonte: a autora.

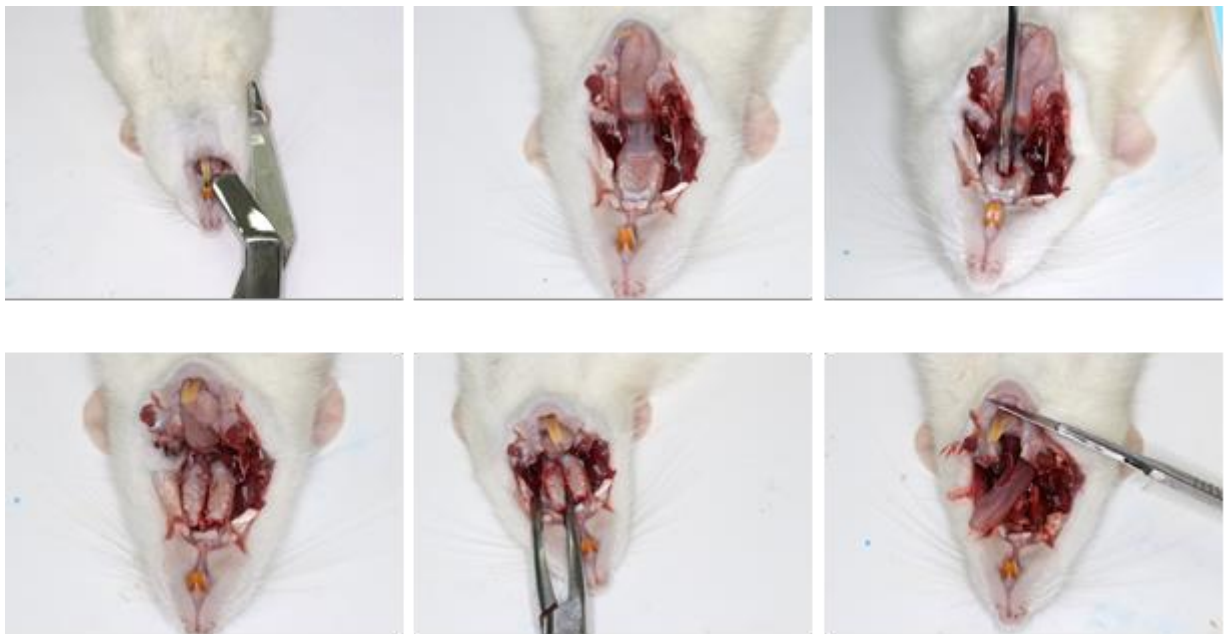


Figura 11 – Sequência de remoção das hemiarcadas para as análises.
Fonte: a autora.

4.3.3. Microscopia eletrônica de varredura

Espécimes representativos dos grupos apresentados na Tabela 2, e do grupo BLE+B-NEG150, no período de avaliação de 24 horas, seguindo os protocolos citados, foram selecionados aleatoriamente e mantidos em temperatura ambiente em cera utilidade por 48 horas para desidratação (Figura 12). Posteriormente, os espécimes foram posicionados em um suporte e pulverizados com ouro (Bal TEc, SCD 050, Balzers Union Aktiengesellschaft, Balzers, Liechtenstein) a fim de

melhorar o nível de emissão de elétrons e facilitar a construção da imagem⁶⁶ (Figura 13).

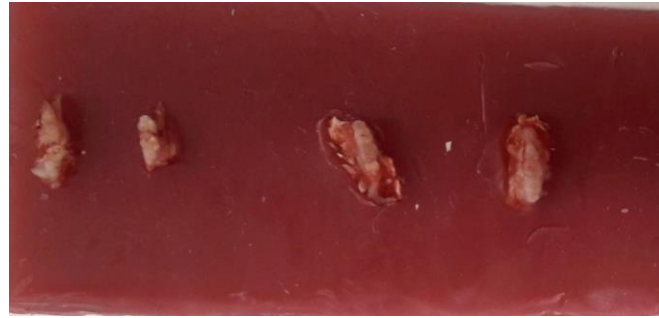


Figura 12 – Desidratação dos espécimes para análise de MEV.
Fonte: a autora.

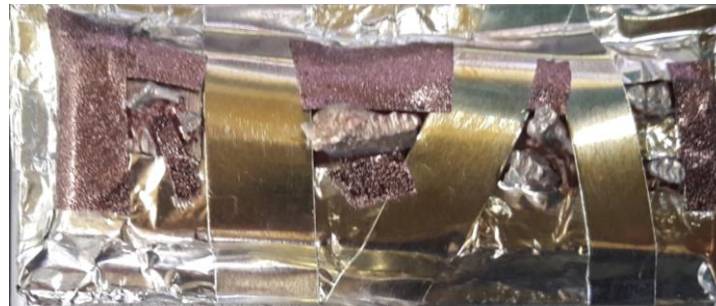


Figura 13 – Posicionamento dos espécimes em suporte e revestimento com ouro para análise de MEV.
Fonte: a autora.

As alterações na superfície do esmalte foram analisadas qualitativamente através de um microscópio eletrônico de varredura (Auriga SEM-FEG, Carl Zeiss Microscopy, Alemanha) equipado com detector de elétrons secundários em baixo vácuo (Figura 14), disponibilizado pelo Laboratório de Caracterização Estrutural de Materiais/Engenharia de Materiais/UFRN. As imagens foram registradas para cada espécime em magnificações de 5000x, 10000x e 20000x. A resolução das imagens foi de 1280 x 1024 pixels.

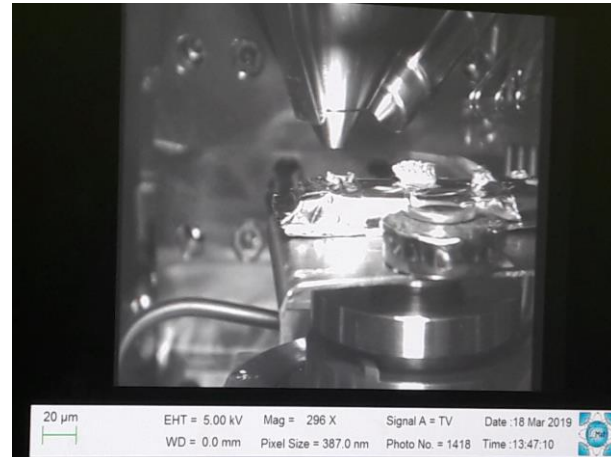


Figura 14 - Microscópio eletrônico de varredura (MEV-FEG, Carl Zeiss Microscopy, Auriga, Alemanha) com detector de elétrons secundários em baixo vácuo.
Fonte: a autora.

4.3.4. Microscopia de fluorescência e microscopia confocal de varredura a laser

O hidrogel e o nanogel carregados com IBF, bem como o nanogel branco, foram marcados com rodamina B na concentração de 0,1% (Sigma-Aldrich, St. Louis, EUA) (Figura 15) para observar suas penetrações através do esmalte e capacidade de atingir a polpa dentária⁶⁷⁻⁶⁹. Este agente fluorescente apresenta absorção e transmissão máximas de aproximadamente 540 nm e 625 nm, respectivamente⁷⁰. Os animais foram submetidos aos mesmos protocolos de aplicação descritos para os grupos na Tabela 2 no período de avaliação de 24 horas. Os espécimes foram armazenados em óleo de soja (Liza, Cargill Brasil, Brasil) por 48 horas a 4°C para minimizar a dissolução do agente fluorescente⁷¹.

Após o período de armazenamento, cada hemiarcada foi fixada com cera 7 (Newwax, Technew, Brasil), em um dispositivo confeccionado em resina acrílica (Figura 16), e cada elemento dentário foi cortado no sentido vestibulo-lingual com disco diamantado a 200 rpm (Isomet; Buehler, Lake Bluff, IL, EUA) (Figura 17) sob lubrificação com óleo. Este procedimento foi realizado no Laboratório de Cimentos / Núcleo Tecnológico em Cimentação de Poços de Petróleo do Instituto de Química / UFRN.

Para montagem das lâminas, os espécimes (0,75 mm de espessura) foram posicionados em cera utilidade (Newwax, Technew, Brasil) (Figura 18) e analisados

através do microscópio reto de fluorescência e campo claro Zeiss Imager A.2 (Figura 19) com ampliações de 5x e 20x, e do microscópio confocal de varredura a laser Zeiss LSM 710 (Carl Zeiss, Jena, Alemanha) (Figura 20) com ampliação de 10x e resolução de 1024 x 1024 pixels. Ambos os equipamentos são pertencentes ao Laboratório Multiusuário de Microscopia/Instituto do Cérebro/UFRN.

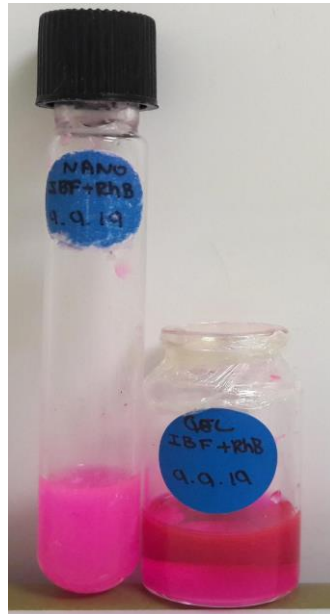


Figura 15 – Nanogel termorresponsivo de ibuprofeno (frasco da esquerda) e Hidrogel convencional de ibuprofeno (frasco da direita) carregados com rodamina B. As substâncias foram confeccionadas no Laboratório de Tecnologia Farmacêutica e Biotecnologia/Departamento de Farmácia/UFRN.

Fonte: a autora.



Figura 16 – Fixação das hemiarcadas em dispositivo confeccionado em resina acrílica.

Fonte: a autora.

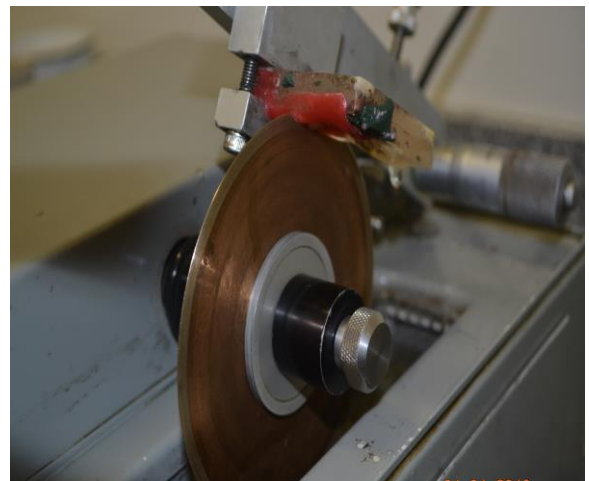


Figura 17 - Corte dos elementos dentários com disco diamantado a 200 rpm (Isomet; Buehler, Lake Bluff, IL, USA).

Fonte: a autora.



Figura 18 – Montagem das lâminas para análises na microscopia de fluorescência e microscopia confocal de varredura a laser.
Fonte: a autora.



Figura 19 - Microscópio reto de fluorescência e campo claro Zeiss Imager A.2.
Fonte: a autora.

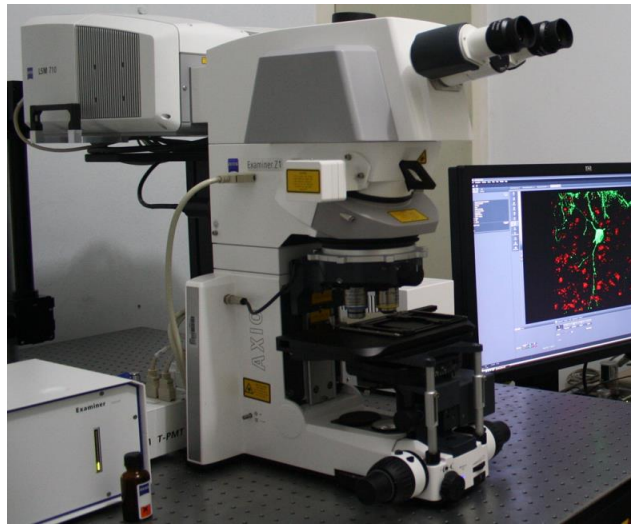


Figura 20 - Microscópio confocal de varredura a laser Zeiss LSM 710 (Carl Zeiss, Jena, Alemanha)
Fonte: a autora.

4.3.5. Análise morfológica

Para a análise morfológica, os maxilares foram descalcificados com EDTA 10% (sal dissódico PA, ACS, Dynamic, Brasil) por 90 dias. Em seguida, esses foram cortados macroscopicamente no sentido méso-distal, posicionados em cassetes histológicos (Figura 21), desidratados em uma série graduada de etanol e incorporados em parafina (Figura 22). Secções com espessura de 5 μm foram obtidas (micrótomo Leica RM2125RT, Leica Biosystems, Nussloch, Alemanha) (Figura 23) e coradas pela técnica da hematoxilina-eosina. A análise morfológica dos tecidos foi realizada por dois patologistas orais calibrados através do microscópio óptico Nikon E200 (Nikon Corporation, Tóquio, Japão) sob aumentos de 100x e 400x. Os procedimentos foram realizados no Laboratório de Anatomia Patológica e Citopatologia da Disciplina de Patologia Oral do Departamento de Odontologia/UFRN. A polpa coronária foi dividida em terços (oclusal, médio e cervical), adaptado de Cintra et al.²³. Foram avaliados os seguintes parâmetros morfológicos: necrose; hiperemia; degeneração vacuolar; perda e desorganização da camada odontoblástica; formação de dentina terciária; presença de calcificações e intensidade do infiltrado inflamatório. Os escores atribuídos à intensidade do infiltrado inflamatório foram adaptados de Cintra et al.²³: 1 = ausência de células inflamatórias ou número desprezível; 2 = infiltrado inflamatório leve (até 25% das células por campo); 3 = infiltrado inflamatório moderado (entre 25% e 75% das células por campo); 4 = infiltrado inflamatório severo (> 75% células por campo) ou necrose.

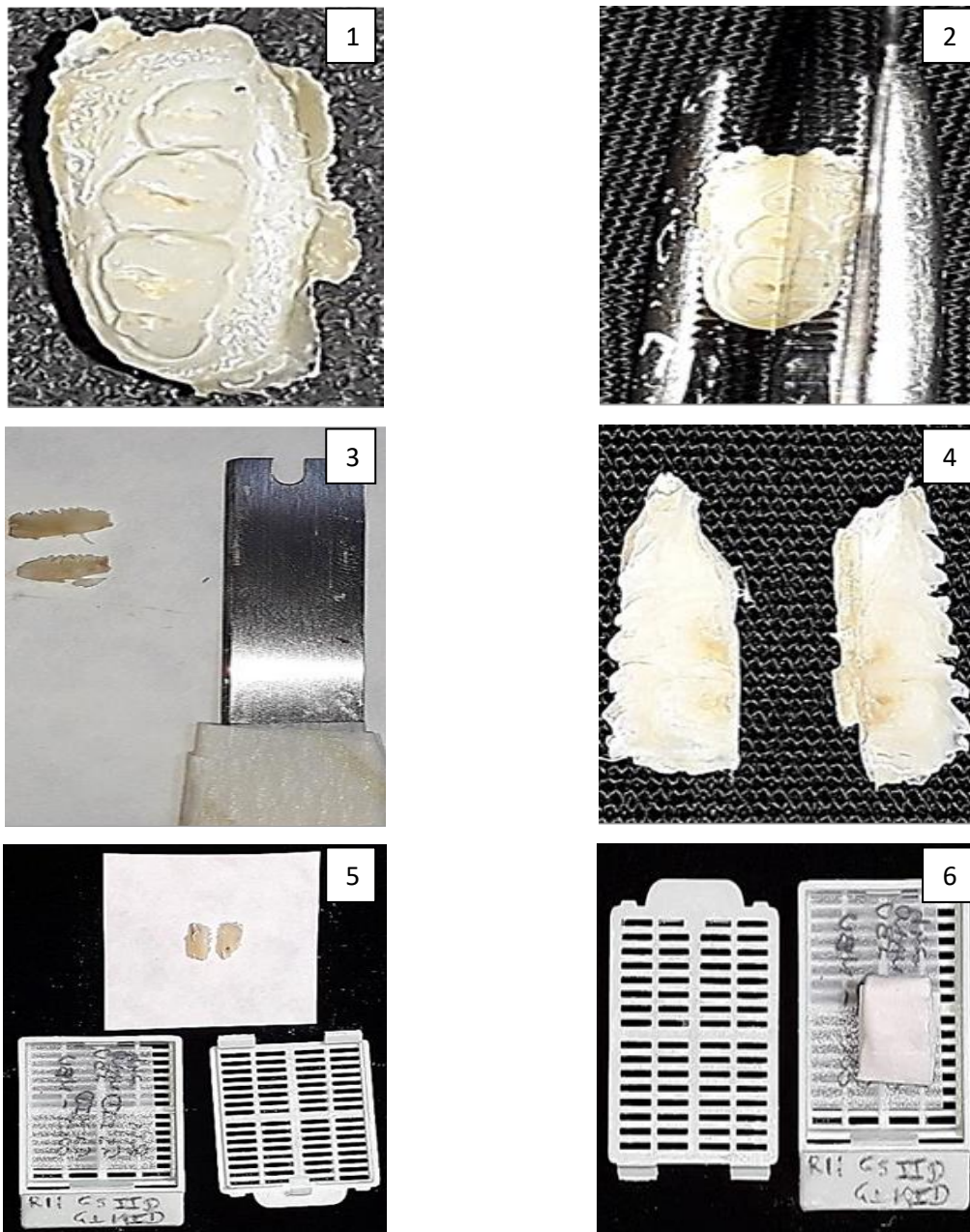


Figura 21 – (1 a 6) Sequência do corte macroscópico das hemiarquadas no sentido méso-distal e posicionamento em cassete histológico.
Fonte: a autora.

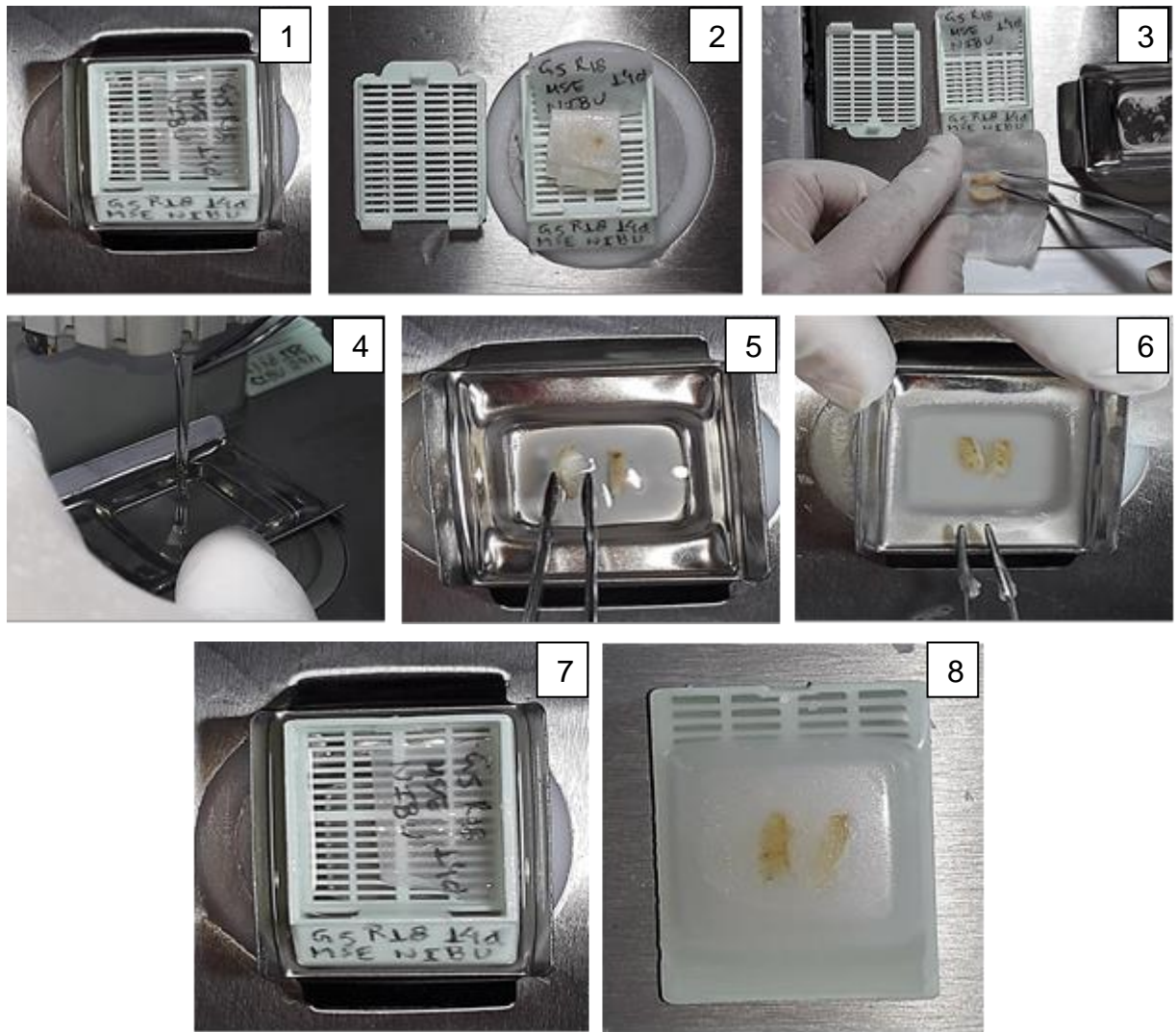


Figura 22 – (1 a 8) Sequência de inclusão dos espécimes em parafina.
Fonte: a autora.

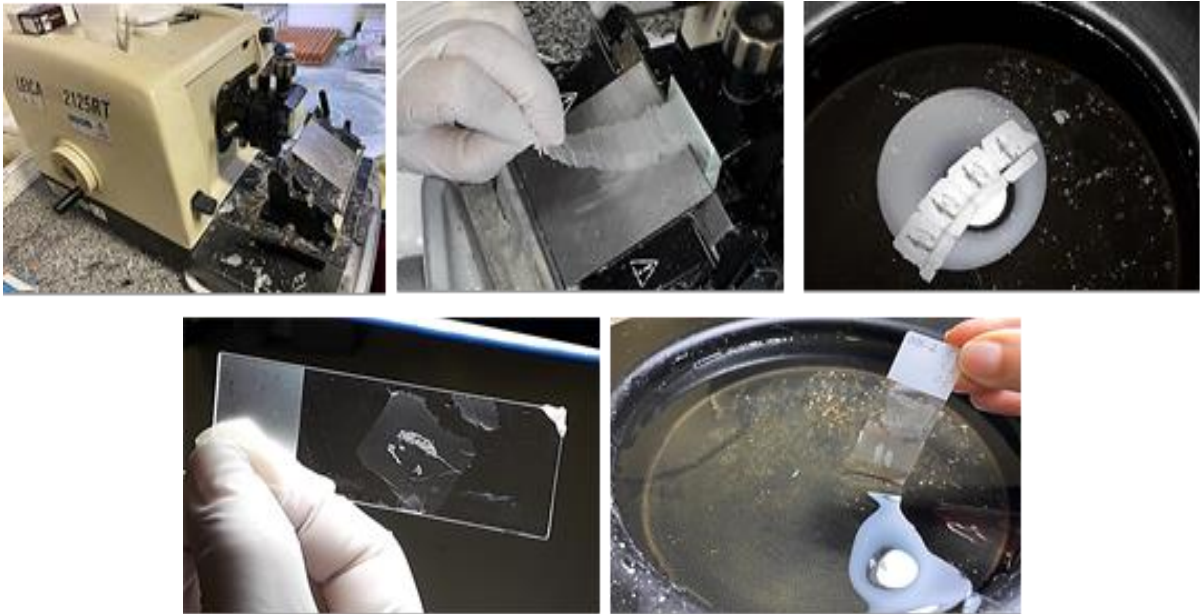


Figura 23 – Corte dos espécimes em micrótomo (Leica RM2125RT, Leica Biosystems, Nussloch, Alemanha) para obtenção de cortes com espessura de 5 μm (análise morfológica) e de 3 μm (análise imuno-histoquímica)

Fonte: a autora.

4.3.6. Análise imuno-histoquímica

Para o estudo imuno-histoquímico, cortes de 3 μm foram obtidos de blocos de tecido incluídos em parafina (Figura 23). A recuperação antigênica, diluição do anticorpo e tipo clonal de cada anticorpo estão listados na Tabela 3. Os cortes foram submetidos ao método da imuno-histoquímica pela técnica da estreptavidina-biotina. Estes procedimentos foram realizadas no Laboratório de Investigação do Câncer e Inflamação (LAICI) do Departamento de Morfologia/UFRN. Secções de gânglio trigeminal de rato foram usadas como controle positivo. Como controle negativo, as amostras foram tratadas conforme descrito anteriormente, exceto para o anticorpo primário que foi substituído por albumina de soro bovino em solução tampão. A intensidade da imunomarcção do tecido pulpar foi visualizada sob aumento de 100x através do microscópio óptico Nikon E100 (Nikon Corporation, Tóquio, Japão). Estas análises foram realizadas no Laboratório de Anatomia Patológica e de Citopatologia do Departamento de Odontologia/UFRN por dois patologistas orais calibrados nos terços da polpa coronária (oclusal, médio e cervical)²³ e avaliada utilizando escores pré-estabelecidos adaptados de Benetti et al.²⁰: 1 = ausência de imunomarcção (ausência de marcação na matriz extracelular e ausência completa de células imunorreativas); 2 = baixa imunoexpressão (fraca marcação da matriz extracelular e aproximadamente um quarto das células imunorreativas); 3 = moderada

imunoexpressão (moderada marcação da matriz extracelular e aproximadamente metade das células imunorreativas); 4 = forte imunoexpressão (forte marcação da matriz extracelular e aproximadamente três quartos das células imunorreativas).

Tabela 3. Fabricante, clone, recuperação antigênica, diluição e período de incubação dos anticorpos primários.

Anticorpo	Fabricante	Clone	Recuperação antigênica	Diluição	Incubação
IL-1 β	Santa Cruz Biotechnology	sc-32294	Trilogy	1:300	Overnight a 4°C
IL-10	Santa Cruz Biotechnology	sc-8438	Trilogy	1:300	Overnight a 4°C
COX-2	Santa Cruz Biotechnology	sc-19999	Trilogy	1:300	Overnight a 4°C
Substância P	Santa Cruz Biotechnology	sc-58591	Trilogy	1:400	Overnight a 4°C
Bradicinina	Santa Cruz Biotechnology	sc-25484	Trilogy	1:300	Overnight a 4°C

4.4. Análise estatística

Os coeficientes de concordância interexaminador para as variáveis qualitativas ordinais (Kappa ponderado) foram calculados como: $\kappa = 0,730$ para o grau de infiltrado inflamatório; $\kappa = 0,870$ para os escores de IL-10; $\kappa = 0,690$ para os escores de IL-1 β ; $\kappa = 0,774$ para os escores de SP; $\kappa = 0,929$ para escores de bradicinina; e $\kappa = 0,830$ para escores de COX-2, mostrando uma concordância substantiva ou quase perfeita. Os dados foram testados quanto à normalidade e homocedasticidade. O teste não paramétrico de Mann-Whitney foi utilizado para analisar os escores do infiltrado inflamatório dentro de cada grupo de acordo com o tempo (24 horas e 14 dias). O teste não paramétrico de Kruskal-Wallis, seguido pelo pós teste de Dunn e análise de variância unilateral, foi aplicado para comparações individuais de escores entre os grupos. Os dados correspondentes aos terços da polpa coronária de cada grupo foram tabulados. Adotou-se nível de significância $\alpha = 0,05$ para todos os testes.

5. ARTIGO PRODUZIDO

O artigo intitulado '*Design of a thermosensitive ibuprofen-loaded nanogel as smart material applied as anti-inflammatory agent in tooth bleaching: An in vivo study*' foi aceito para publicação no periódico 'Journal of Drug Delivery Science and Technology' que possui fator de impacto 3.981.

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Opinion paper

Design of a thermosensitive ibuprofen-loaded nanogel as smart material applied as anti-inflammatory in tooth bleaching: An *in vivo* study



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ABSTRACT

The *in vivo* ability of thermosensitive nanogel containing ibuprofen for bioadhesion on the surface of teeth submitted to bleaching and drug delivery through dentinal tubules to the dental pulp are evaluated in Wistar rats. Drug-loaded self-assembled oil in water nanoemulsions present droplet size <150 nm and negative zeta potential. Nanoemulsions containing poloxamer 407 in the aqueous phase exhibit reduced droplet size, improved physical stability, controlled the thermosensitive viscosity, and slow drug release. Different treated animals' groups included a control group (CTRL); bleached animals (BLE) (37.5% H₂O₂, Pola Office Plus®, SDI); bleached animals treated with a conventional ibuprofen-loaded hydrogel (BLE + IBF-GEL), and bleached animals treated with thermosensitive ibuprofen-loaded nanogel (BLE + IBF-NEG). Compared to the BLE + IBF-GEL group, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) images show the superior ability of BLE + IBF-NEG group to offer a physical barrier on the tooth surface, pores occlusion, and transport the fluorescent dye rhodamine B to dental pulp. Morphological analysis revealed the lowest inflammatory infiltrate scores and absence of necrosis for BLE + IBF-NEG group after 24 h and 14 days. Immunohistochemical studies reinforced this hypothesis. The experimental data presented in this study confirm the incredible potential for topical and easy application of a novel and smart nanogel, as analgesic and anti-inflammatory treatment for bleaching procedure.

1. Introduction

Esthetic solutions are attracting increasing investments from the dental industry. Tooth bleaching is an effective and conservative esthetic treatment when well indicated [1–3]. However, this treatment

remains a challenge in terms of sensitivity control. Tooth bleaching directly affects the organic components of dental tissues and promotes changes in the mineral phase that result in visible morphological surface alterations [4–6]. The increase in porosity and exposure of Tomes' processes are suggestive of increased sensitivity [7].

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Sensitivity is reported in tooth bleaching mainly due to the use of hydrogen peroxide (H_2O_2) at concentrations close to 35% [8–10]. The H_2O_2 and its degradation products (reactive oxygen species) show a high potential for expose pores on the teeth surface accessing dentinal tubules and penetration into the tooth because of their low molecular weight [11,12], whitening teeth by oxidizing the organic enamel matrix [13] and possibly by oxidation of aromatic amino acids present in dentin phosphoprotein [14].

The evaluation of inflammatory mediators used to map pulp changes after exposure to bleaching agent is fundamental to elucidate how this relationship is established. Some studies have observed an increase in the expression of proinflammatory cytokines, cyclooxygenase 2 (COX-2) and interleukin 1 beta (IL-1 β), in the pulp of the groups submitted to tooth bleaching compared to the control [15,16], as well as in the neuropeptide, substance P (SP) [17,18]. Higher levels of bradykinin, as well as of the anti-inflammatory cytokine interleukin-10 (IL-10), have been identified in human pulp tissue diagnosed with irreversible pulpitis [19,20]; however, the expression of these substances during tooth bleaching is still unknown.

In an effort to identify alternatives that can prevent the sensitivity caused by bleaching, some human studies have shown that systemic administration of ibuprofen (IBF) reduces symptoms during treatment or even up to the first hour after bleaching [21]. Furthermore, recent animal studies have reported positive results for the use of anti-inflammatory substances applied topically to the enamel after bleaching treatment, such as otosporin and carvedilol, minimizing pulp damage caused by bleaching [18,22,23].

Ibuprofen (RS-2-(4-(2-methylpropyl) phenyl) propanoic acid) is a non-selective inhibitor of cyclooxygenases (COX-1 and COX-2), enzymes involved in prostaglandin synthesis. It is a crystalline and non-thermolabile drug [24,25], with low solubility in water (0.02–0.09 mg/mL) [26], which limits the oral bioavailability. In addition, the rapid degradation in the stomach, and considerable first-pass metabolism are additional challenges for developing an alternative and efficient topical dosage form [27].

Nanotechnology alternatives as nanoemulsions (NEs) have demonstrated superior potential for topical penetration when compared to conventional systems [28]. The oil-in-water (O/W) NEs are capable of encapsulating hydrophobic drugs, such as IBF, in oil droplets (100–600 nm) dispersed in water [28–31]. These nanocarriers can penetrate into micrometric channels as the dentinal tubules and transport drug to target tissue controlling drug release and enhancing its efficacy [32,33]. Despite these advantages, NEs have low viscosity, a fact that limits their use in topical dosage forms in dentistry. This limitation can be solved by using hydrogels in the aqueous phase, improving bioadhesion [34]. The use of a thermosensitive copolymer, such as poloxamer 407, seems an interesting alternative for *in situ* increase of NE viscosity and formation of a nanogel (NEG) on the tooth surface, improving the *in vivo* bioadhesion of colloidal dispersion. This thermosensitive NEG provides incredible practical gains when compared to liquid dispersion and conventional hydrogels [35,36].

Therefore, the present study aims to develop an innovative formulation of thermosensitive IBF-loaded nanogel able to be used as topical analgesic and anti-inflammatory approach in tooth bleaching and to evaluate *in vivo* its bioadhesion and ability for penetration through dentinal tubules and drug transport to molar pulp of Wistar rats. A careful evaluation of compositions able to induce self-assembled thermosensitive IBF-loaded nanogel is performed. As null hypothesis: the IBF-loaded nanogel is unable to reduce inflammation in the pulp tissue when applied before and after dental bleaching.

2. Materials and methods

2.1. Materials

Ibuprofen was purchased from All Chemistry (Jabaquara, Brazil).

Medium chain triglyceride (Miglyol 812®) was purchased from Sasol (Hamburg, Germany), soybean phosphatidylcholine (95%) (SPC) was obtained from Avanti Polar Lipids (Alabama, USA), polysorbate 80 (PO80; Tween 80®), and poloxamer 407 (P407; Pluronic F127®) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and propylene glycol was purchased from Qhemis (São Paulo, Brazil). Purified water was obtained using a reverse osmosis system (model OS50 LX, Gehaka Ins. Ltd, Brazil). The other reagents and products used were of analytical grade.

2.2. Preparation of thermosensitive IBF-loaded nanogel

2.2.1. Ibuprofen solubility test

The solubility of IBF was tested in different ingredients used in the formulations (Miglyol 812®, soybean phosphatidylcholine, Tween 80® and propylene glycol). An excess of drug was mixed with 2 mL of each raw material under constant magnetic stirring (360 rpm) for 48 h at 25 °C, adapted from Dukovski et al. [37]. Dispersions were centrifuged for 5 min at 5000 rpm. The volume taken (1 mL) was diluted to 10 mL in volumetric flasks with ethanol [38]. The absorbance was measured ($\lambda = 264$ nm) and the drug content was calculated using the equation ($y = 0.0017x - 0.0143$; $r^2 = 0.9999$) from of a standard curve constructed under the same analytical conditions, considering analytical validation parameters [39].

2.2.2. Preparation of nanoemulsions

Nanoemulsions were prepared by the phase inversion method [31]. The purified water was slowly (1 mL/mL) dropped into the oil phase containing a blend of soybean oil, surfactant mixture (SM; SPC:PO80, 3:1 w/w) and the co-solvent propylene glycol, at 65 °C, under magnetic stirring at 1500 rpm for 40 min. The temperature of 65 °C was used considering the melting point of drug (of about 80 °C) and thermal stability at this range [24,25]. The basic formulations consisted of 1% (w/w) soybean oil and 10% (w/w) propylene glycol. The SM in the formulations ranged from 1% (w/w) to 5% (w/w) and water from 88% (w/w) to 84% (w/w), respectively. The same method was used for IBF-loaded NEs, in which 1% (10 mg/mL) of the drug was dissolved in the oil phase. After preparation, the NEs were filtered and drug loading was measured in the NEs, as described for the IBF solubility test. The detailed composition of the NEs is described in Table 1.

2.2.3. Preparation of nanogels

The thermosensitive nanogels were prepared by dissolving P407 (Pluronic F127®) in the NEs containing 1% of SM; 24 h after NEs preparation as gelling agent at 4 °C under vortex homogenization for 2 min. Different concentrations of P407 were tested (10% w/w, 12.5% w/w, 15% w/w, 17.5% w/w and 20% w/w). For *in vivo* performance comparisons, a conventional IBF-loaded hydrogel was prepared in the absence of NE using 2% hydroxypropyl methylcellulose (HMPC) in water. The detailed composition of the NEGs is described in Table 1.

2.2.4. Droplet size and zeta potential of nanoemulsions

The average droplet size and polydispersity index (PDI) of the formulations were assessed by dynamic light scattering using a Malvern Zetasizer Nano ZS equipment (Malvern Ins. Ltd, UK). The samples were diluted 1:100 in ultrapure water and analyzed at 25 °C at a detection angle of 173° and wavelength of 633 nm. The values obtained are expressed as mean \pm standard deviation ($n = 2$). Zeta potentials were measured using the same equipment, under the same conditions, by assessing electrophoretic mobility. The samples were also diluted 1:100 in ultrapure water.

2.2.5. Physical stability of nanoemulsions

Before the drug-loading experiments, the physical stability of the blank formulations was monitored for 60 days at 25 °C. The drug-loaded NEs were monitored for 15 days under the same conditions. The samples

Table 1
Composition of the different formulations.

Formulations	Composition (% w/w)							
	IBF	Oil	SPC	PO80	Co-solvent	P407	HPMC	Water
B-NE10	0.0	1.0	0.75	0.25	10.0	0.0	0.0	88.0
B-NE25	0.0	1.0	1.875	0.625	10.0	0.0	0.0	85.5
B-NE50	0.0	1.0	3.75	1.25	10.0	0.0	0.0	84.0
IBF-NE10	1.0	1.0	0.75	0.25	10.0	0.0	0.0	87.0
IBF-NE25	1.0	1.0	1.875	0.625	10.0	0.0	0.0	84.5
IBF-NE50	1.0	1.0	3.75	1.25	10.0	0.0	0.0	83.0
B-NEG100	0.0	0.0	0.75	0.25	10.0	10.0	0.0	78.0
B-NEG125	0.0	0.0	0.75	0.25	10.0	12.5	0.0	75.5
B-NEG150	0.0	0.0	0.75	0.25	10.0	15.0	0.0	73.0
B-NEG175	0.0	0.0	0.75	0.25	10.0	17.5	0.0	70.5
B-NEG200	0.0	0.0	0.75	0.25	10.0	20.0	0.0	68.0
IBF-NEG100	1.0	1.0	0.75	0.25	10.0	10.0	0.0	77.0
IBF-NEG125	1.0	1.0	0.75	0.25	10.0	12.5	0.0	74.5
IBF-NEG150	1.0	1.0	0.75	0.25	10.0	15.0	0.0	72.0
IBF-NEG175	1.0	1.0	0.75	0.25	10.0	17.5	0.0	69.5
IBF-NEG200	1.0	1.0	0.75	0.25	10.0	20.0	0.0	67.0
B-GEL	0.0	0.0	0.0	0.0	0.0	0.0	2.0	98.0
IBF-GEL	1.0	0.0	0.0	0.0	0.0	0.0	2.0	97.0

Note: Ibuprofen (IBF), soybean phosphatidylcholine (SPC), polysorbate 80 (PO80), poloxamer 407 (P407), hydroxypropyl methylcellulose (HPMC), blank nanoemulsion (B-NE), IBF-loaded nanoemulsion (IBF-NE), blank nanogel (B-NEG), IBF-loaded nanogel (IBF-NEG), blank hydrogel (B-GEL), IBF-loaded hydrogel (IBF-GEL).

were stored in hermetically closed glass flasks. The average droplet size, Pdl, and zeta potential were measured as described in item 2.2.4.

2.2.6. Drug-loading assay

The amount of 0.5 g of the formulations was dissolved in 2 mL methanol and completed to 10 mL with ethanol in volumetric flasks [40]. The filtered solutions were analyzed in a UV-Vis spectrophotometer at 264 nm, as described for the IBF solubility test.

2.2.7. In vitro release study

The *in vitro* release of the drug was studied using vertical Franz diffusion cells at 37 °C [41]. The receptor compartment was filled with phosphate-buffered saline (PBS; pH = 7.4; 11 mL ± 1) kept under constant agitation (100 rpm) and separated from the donor compartment by a 0.45-µm acetate membrane (previously hydrated in PBS for 24 h). At specific time intervals, 1-mL aliquots were removed for drug analysis by UV-Vis spectrophotometry at 264 nm. The same volume of PBS was replaced to the medium immediately thereafter to maintain the sink conditions.

2.3. In vivo studies

2.3.1. Animals

The experimental protocol followed the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) [42] for animal research suggested by the National Center for Replacement, Refinement, and

Reduction for Animals in Research. All animal protocols were approved by the Institutional Research Ethics Committee (CEUA, Protocol No. 037/2016). The animals were housed in a temperature-controlled environment (22 ± 1 °C, 70% humidity) on a standard light/dark schedule, with access to food and water ad libitum. Male rats (*Rattus norvegicus*) (n = 48) weighing between 250 and 300 g were used in this split-mouth study. Of these, 30 animals were used for morphological and immunohistochemical analysis. The rats were randomly assigned to four groups (five animals per group): CTRL group, BLE group, BLE+IBF-GEL group, and BLE+IBF-NEG group, and evaluated after 24 h and 14 days. The upper and lower molars were used in the split-mouth study and in all groups. The left molars were used for the experimental groups, while the right molars were used as control group, totaling n = 10 hemi-arches analyzed per group. The thermosensitive IBF-loaded nanogel selected for the *in vivo* studies was the IBF-NEG150 formulation (Table 2; Fig. 1). All performed *in vivo* experiments used freshly prepared formulations (interval of 24 h) and drug-loaded sample have the analytical drug content determined.

Initial experiments were carried out on four animals (BLE+B-NEG150 group). Of these, two animals were analyzed after 24 h, and two animals were analyzed after 14 days by applying the blank nanogel (B-NEG150) to the upper and lower left molars, totaling eight hemi-arches evaluated. The standard application for the BLE+IBF-NEG group was followed to analyze possible interference of the presence of IBF with the performance of the substance under analysis. The small number of animals did not allow to carry out statistical analysis (Supplementary

Table 2
Treatment groups and application protocols.

Group	Description	Substances	Application protocol	24-h evaluation period	14-day evaluation period
CTRL	Control	None	None	5 animals ^a (n = 10 hemi-arches)	5 animals ^a (n = 10 hemi-arches)
BLE	Bleached	37.5% H ₂ O ₂ (Pola Office Plus®, SDI)	3 applications (8 min each)	5 animals (n = 10 hemi-arches)	5 animals (n = 10 hemi-arches)
BLE + IBF-GEL	Bleached animals treated with a conventional IBF-loaded hydrogel	37.5% H ₂ O ₂ IBF-GEL (Composition specified in Table 1)	IBF-GEL + 37.5% H ₂ O ₂ ^b + IBF-GEL	5 animals (n = 10 hemi-arches)	5 animals (n = 10 hemi-arches)
BLE+IBF-NEG	Bleached animals treated with thermosensitive IBF-loaded nanogel	37.5% H ₂ O ₂ IBF-NEG150 (Composition specified in Table 1)	IBF-NEG150 + 37.5% H ₂ O ₂ ^b + IBF-NEG150	5 animals (n = 10 hemi-arches)	5 animals (n = 10 hemi-arches)

Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group.

^a The animals of the control group were randomly chosen among animals of the experimental groups in a split-mouth design.

^b The bleaching agent was applied to all groups following the same protocol as described for BLE group.

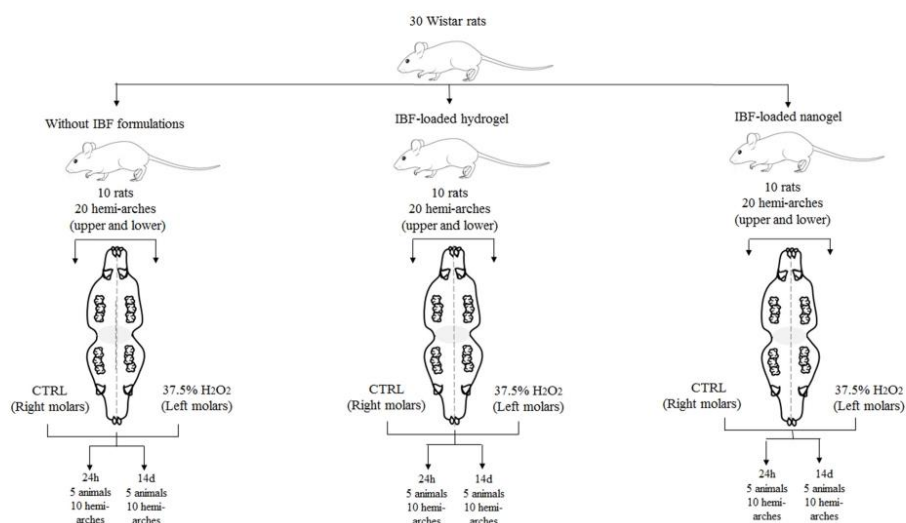


Fig. 1. Flowchart of animal allocation to the study groups. Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group.

material).

The other animals were randomly divided for qualitative analysis by scanning electron microscopy (SEM), fluorescence microscopy, and confocal laser scanning microscopy (CLSM). The protocols were carried out under intraperitoneal anesthesia with 10% ketamine hydrochloride (Syntec, Brazil; 70 mg/kg) and 2% xylazine hydrochloride (Syntec, Brazil; 7 mg/kg). All animals were weighed prior to the start of the procedures and euthanasia, which was performed with an overdose of intraperitoneal sodium thiopental (100 mg/kg).

2.3.2. Tooth bleaching and anti-inflammatory protocols

All experimental groups received applications of 37.5% H₂O₂ bleaching agent (Pola Office Plus®, SDI, Australia) and the periodontal tissues were protected with a resinous gingival barrier (Top Dam, FGM, Brazil) according to the manufacturer's recommendations (Table 2). Animals of the BLE+IBF-GEL and BLE+IBF-NEG groups (Table 2) received applications of the respective IBF-loaded formulations 10 min before the application of bleaching agent and 10 min after its removal, adapted from Gallinari et al. [18]. The substances were applied with the aid of a microbrush using an approximate volume of 0.01 mL per hemi-arch. All products applied to the teeth were removed with an aspirating cannula and the teeth were washed with water. The procedures were performed by a dentist.

After application of the protocols, the animals were observed in a postoperative control room and were euthanized according to the evaluation periods. The hemi-arches were removed and stored in a 10% buffered formaldehyde fixative solution for morphological and immunohistochemical analysis.

2.3.3. Scanning electron microscopy

Specimens representative of the groups shown in Table 2 for the 24-h evaluation period (as well as the BLE+B-NEG150 group), following the aforementioned protocols, were randomly selected and kept at room temperature for 48 h for dehydration. Changes in the enamel surface were analyzed qualitatively using a scanning electron microscope (Auriga SEM-FEG, Carl Zeiss Microscopy, Germany) equipped with a secondary electron detector at low vacuum. Images were recorded for each specimen at magnifications of 5,000 \times , 10,000 \times and 20,000 \times in

order to determine changes in the enamel surface. The images resolution was 1280 \times 1024 pixels.

2.3.4. Fluorescence microscopy and confocal laser scanning microscopy

The IBF-loaded hydrogel and IBF-loaded nanogel were doped with 0.1% rhodamine B (Sigma-Aldrich, St. Louis, USA), as well as the blank nanogel, to observe their penetration into the tooth enamel reaching the dental pulp [43,44]. The animals were submitted to the same application protocols as described for the groups in Table 2 for the 24-h evaluation period. The specimens were stored in soybean oil (Liza, Cargill Brasil, Brazil) for 48 h at 4 °C to minimize dissolution of the fluorescent agent [45]. After the storage period, each tooth was cut in the buccal-lingual direction with a diamond disc at 200 rpm (Isomet; Buehler, Lake Bluff, IL, USA) under oil lubrication. The specimens (0.75-mm thick) were mounted on slides and analyzed under a Zeiss Imager A.2 fluorescence and brightfield microscope at magnifications of 5 \times and 20 \times and under a Zeiss LSM 710 confocal microscope system (Carl Zeiss, Jena, Germany) at 10 \times magnification using a pixel resolution of 1024 \times 1024.

2.3.5. Morphological analysis

Morphological analysis of the tissues was performed under a Nikon E200 light microscope (Nikon Corporation, Tokyo, Japan) at 100 \times and 400 \times magnification. Morphological examination of the dental tissues was conducted by two calibrated oral pathologists. The coronal pulp was divided into thirds (occlusal, middle and cervical), adapted from Cintra et al. [46]. The following morphological parameters were evaluated: necrosis; hyperemia; vacuolar degeneration; loss and disorganization of the odontoblastic layer; tertiary dentin formation; presence of calcifications, and inflammatory infiltrate intensity. The scores attributed to the intensity of the inflammatory infiltrate were adapted from Cintra et al. [46]: 1 = absence of inflammatory cells or negligible number; 2 = mild inflammatory infiltrate (up to 25% of cells per field); 3 = moderate inflammatory infiltrate (between 25% and 75% of cells per field); 4 = severe inflammatory infiltrate (>75% cells per field) or necrosis.

2.3.6. Immunohistochemical analysis

The antigen retrieval, antibody dilution, and clone type of each

antibody are listed in Table 3. The immunostaining intensity in pulp tissue was visualized at 100× magnification (Nikon E100, Nikon Corporation, Tokyo, Japan). Immunohistochemical analysis was performed by two calibrated oral pathologists on coronal pulp thirds (occlusal, middle and cervical) [46]. Immunostaining was evaluated using pre-established scores adapted from Benetti et al. [22]: 1 = absence of immunostaining (absence of staining in the extracellular matrix and complete absence of immunoreactive cells); 2 = low immunoreactive cells; 3 = moderate immunoreactive cells; 4 = strong immunoreactive cells (strong staining of the extracellular matrix and approximately three quarters of immunoreactive cells).

2.4. Statistical analysis

The non-parametric Mann-Whitney test was used to analyze the inflammatory infiltrate scores within each group according to time (24 h and 14 days). The non-parametric Kruskal-Wallis test, followed by Dunn's post hoc test and one-way analysis of variance, was applied to individual comparisons of scores between groups. The data corresponding to the coronal pulp third of each group were tabulated. A level of significance of $\alpha = 0.05$ was adopted for all tests.

3. Results

3.1. Preparation of thermosensitive IBF-loaded nanogel

The solubility values assessed for IBF in different compounds used for preparing NE formulations was 40.1 mg/mL in medium chain triglyceride, 43.3 mg/mL in propylene glycol, and 13.6 mg/mL in PO80, respectively. Soybean phosphatidylcholine was not used due to its solid state. The amount of oil was set at 1% (w/w) for all formulations and the effect of the amount of surfactant on droplet size was evaluated (Fig. 2). This experiment was also performed using NE formulations containing 1% (w/w) of the drug before and after P407 addition to obtain NEs.

Fig. 2A and B shows how the surfactant affected droplet size and PDI of blank NEs and drug-loaded NEs. It is possible to observe a positive correlation. Drug-loading increased droplet size and appeared to have decreased PDI. Fig. 2C clearly shows that addition of the copolymer decreased the droplet size of both blank and drug-loaded NEs. However, a considerable increase in copolymer ratio seems to increase PDI, mainly for the drug-loaded formulations (Fig. 2D). Fig. 2E and F illustrates the appearance of blank NE and drug-loaded NE. Finally, Fig. 2G demonstrates the successful preparation of NEG using 15% P407, which was considered sufficient to obtain a gelling formulation at room temperature (25 °C) that is suitable for bioadhesion in the oral cavity and teeth.

All blank and drug-loaded NE and NEG formulations exhibited a

negative zeta potential in the range of -15 to -30 mV. There was no correlation of this parameter with the experimental changes evaluated. All drug-loaded formulations were prepared using 1% IBF, including the hydrogel used as control in the *in vivo* experiments. The stability of blank NEs with different concentrations of the SM was evaluated over a period of 60 days at room temperature (Fig. 3A). The zeta potential for all formulations ranged of about -20 mV. It was also observed that a higher concentration of surfactants contributed to increase the droplet diameter and PDI and to decrease the zeta potential.

The formulations containing IBF (Fig. 3B) were evaluated over a period of 15 days at room temperature. After this interval, phase separation was observed before 30 days for samples containing 1% of SM (IBF-NE10) and 2.5% SM (IBF-NE25). The formulation prepared with 5% SM (IBF-NE50) showed phase separation less than 24 h after preparation. The NE formulations (B-NE10 and IBF-NE10) have different added poloxamer ratios (15%, 17.5% and 20% w/w) for producing NEG formulations and their physical stability were also followed for 42 days (Table 4).

The minimal concentration of P407 able to induce NE gelation producing NEG at room temperature (25 °C) was of 15% (w/w). No additional improvement was observed for greater P407 concentrations. The IBF-NEG150 formulation was selected for further studies with *in vitro* drug release experiments (Fig. 3C).

3.2. Scanning electron microscopy

Fig. 4A, 4a1 and 4a2 exhibit SEM images of rat molars collected at magnifications of 5,000×, 10,000×, and 20,000× respectively, considered as CTRL group. It is possible to observe a uniform enamel surface with pore diameters of 64.05–92.38 nm. The specimens submitted to the bleaching treatment (Fig. 4B, b1, and b2) clearly show an increase in roughness and porosity due to demineralization. It is possible to observe a pore diameter of 1.4 μ m (Fig. 4b2), an increase of about 15–20 times, compared to the CTRL group.

The BLE + IBF-GEL group (Fig. 4C, c1, and c2) show the ability to offer occlusion of increased pores on the bleached enamel surface, compared to BLE group. However, arrows in the SEM images highlight the presence of non-soluble or precipitated drug particles. This limitation was not observed for the BLE + IBF-NEG group (Fig. 4D, d1, and d2), suggesting its superior performance compared to conventional hydrogel. Similar distribution pattern to the BLE + IBF-NEG group was observed in the BLE+B-NEG150 group (Supplementary Fig. 1).

3.3. Fluorescence and confocal laser scanning microscopy images

Fig. 5A, 5a1 and 5B, 5b1 show respectively fluorescence and CLSM images of CTRL and BLE groups. It is possible to observe fluorescence due to the aromatic amino acids present in dentin phosphoprotein. This protein is located at the organic-inorganic interface [14]. In addition, it is possible to observe an expressive change in the fluorescence pattern for both samples treated with doped rhodamine, BLE + IBF-GEL (Fig. 5C and c1) and BLE+IBF-NEG groups (Fig. 5D and d1). Comparison of these two last samples showed that the IBF-loaded hydrogel reached the dentinal tubules, but greater amount remained on the tooth surface (please see highlighted field in Fig. 5C and arrow in Fig. 5c1). On the other hand, the IBF-loaded nanogel reached the dentinal tubules and deep layers closer to the dental pulp (please see highlighted field in Fig. 5D and arrows in Fig. 5d1). Similar penetration patterns and fluorescence when compared to the BLE+IBF-NEG group were also observed for the BLE + B-NEG150 group (Supplementary Fig. 2).

3.4. Morphological and immunohistochemical analysis

3.4.1. 24-Hour morphological analysis

Specimens of the CTRL group (no treatment) showed the morphological findings and pulp tissue characteristics compatible with healthy

Table 3
Manufacturer, clone, antigen retrieval, dilution, and incubation period of primary antibodies.

Antibody	Manufacturer	Clone	Antigen retrieval	Dilution	Incubation
IL-1 β	Santa Cruz	sc-	Trilogy	1:300	Overnight at 4 °C
	Biotechnology	32294			
IL-10	Santa Cruz	sc-	Trilogy	1:300	Overnight at 4 °C
	Biotechnology	8438			
COX-2	Santa Cruz	sc-	Trilogy	1:300	Overnight at 4 °C
	Biotechnology	19999			
Substance P	Santa Cruz	sc-	Trilogy	1:400	Overnight at 4 °C
	Biotechnology	58591			
Bradykinin	Santa Cruz	sc-	Trilogy	1:300	Overnight at 4 °C
	Biotechnology	25484			

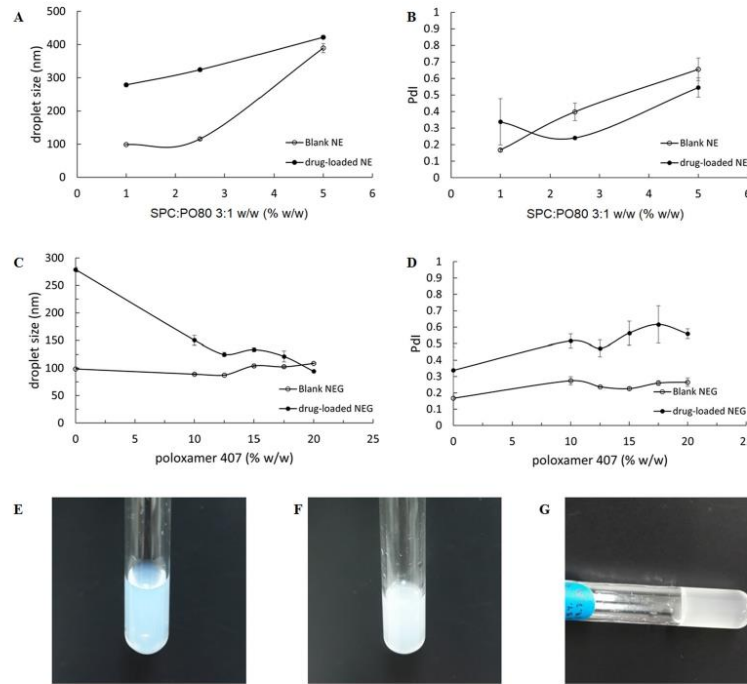


Fig. 2. (A) Droplet size and (B) Pdl of blank and drug-loaded NEs as a function of surfactant ratio (C) Droplet size and (D) Pdl of blank and drug-loaded NEGs as a function of P407 ratio. Clear and translucent appearance of (E) blank NE (1% SM), opaque appearance of (F) drug-loaded NE (1% SM), and apparent viscosity and homogeneous appearance of (G) NEG (15% P407) at 25 °C.

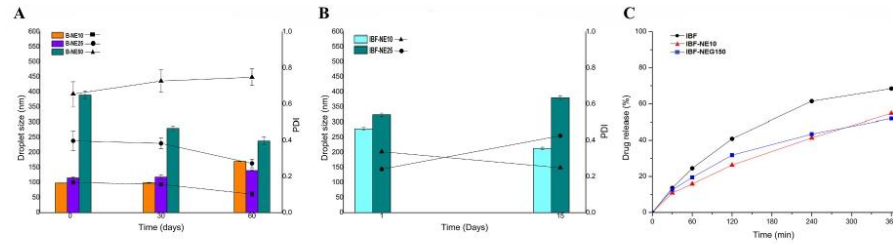


Fig. 3. Evaluation of stability of blank and ibuprofen (IBF) nanoemulsions (NEs) and *in vitro* drug release study. (A) Stability of blank NEs with different concentrations of the surfactant mixture. (B) Stability of NEs with different IBF-containing surfactant mixtures. (C) *In vitro* release study.

Table 4

Assessment of the physicochemical parameters of blank NEG and drug-loaded NEG formulations after 24 h and 42 days.

Sample	24 h			42 days		
	Size (nm)	Pdl	ZP	Size (nm)	Pdl	ZP
B-NEG150	104.1 ± 1.8	0.23 ± 0.01	-25.2 ± 0.2	101.8 ± 2.5	0.32 ± 0.03	-19.8 ± 2.0
B-NEG175	102.5 ± 2.1	0.26 ± 0.01	-23.8 ± 0.1	93.6 ± 1.4	0.39 ± 0.01	-9.1 ± 0.5
B-NEG200	108.8 ± 0.9	0.27 ± 0.02	-30.1 ± 1.2	110.8 ± 11.3	0.33 ± 0.04	-18.7 ± 1.1
IBF-NEG150	134.4 ± 3.7	0.56 ± 0.07	-14.9 ± 0.7	123.9 ± 18.9	0.42 ± 0.07	-17.1 ± 2.2
IBF-NEG175	121.1 ± 9.7	0.62 ± 0.11	-14.8 ± 0.3	129.5 ± 18.7	0.37 ± 0.06	-16.6 ± 1.2
IBF-NEG200	94.2 ± 0.9	0.60 ± 0.03	0.45 ± 0.09	114.1 ± 21.8	0.39 ± 0.04	-18.3 ± 0.7

Note: Polydispersity index (Pdl), zeta potential (ZP).

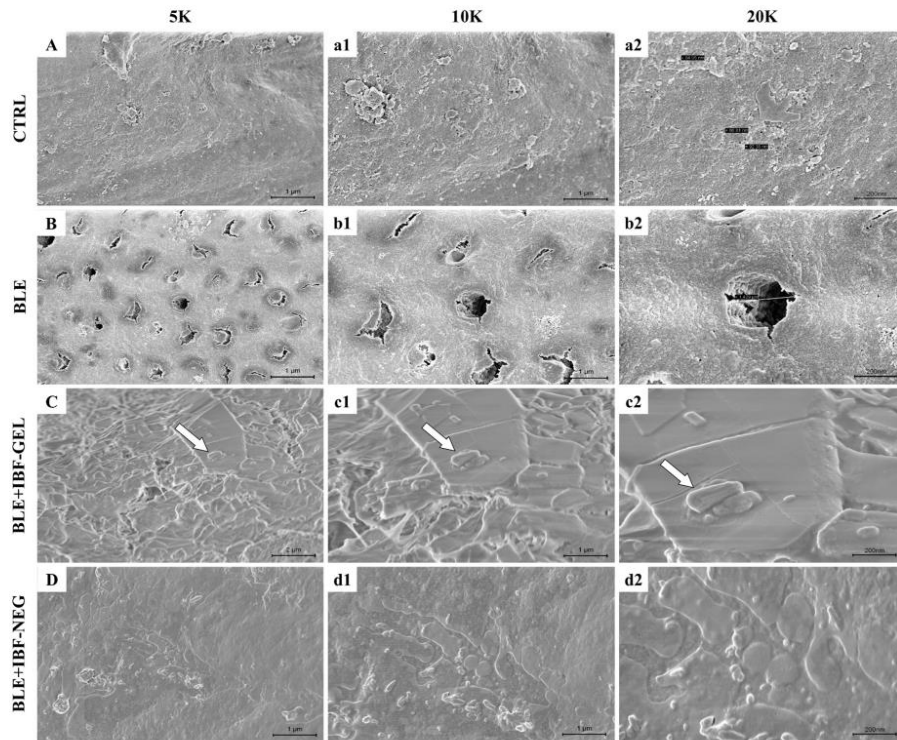


Fig. 4. SEM images of Wistar rat molars. (A-a2) Sound enamel. (B – b2) Increase in enamel pore diameter after exposure to the bleaching agent. (C - c2) Presence of IBF-loaded hydrogel on the tooth surface The precipitate suggests that some hydrogel compound precipitated, possibly the non-solubilized IBF (arrow). (D – d2) Uniform layer of IBF-loaded nanogel on the enamel surface. Note: Image C obtained at larger magnification (2 μm) to demonstrate the suggestive presence of IBF precipitate.

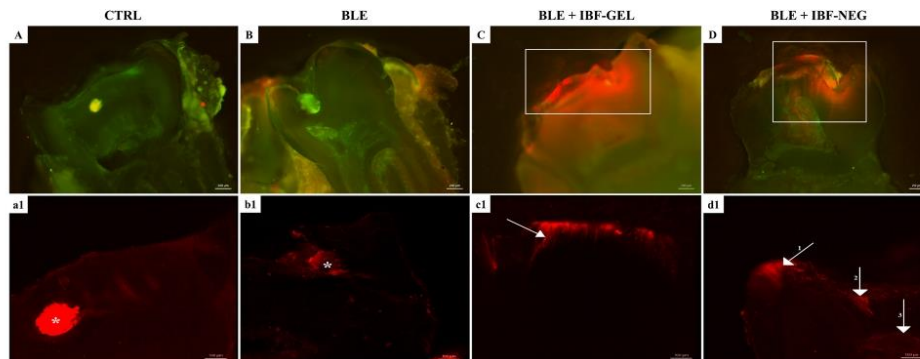


Fig. 5. Drug transport study through dentinal tubules using fluorescence microscopy and confocal laser scanning microscopy for different samples. Representative fluorescence microscopy images of distinct samples (FM; A - D), refers green fluorescence due to dentin phosphoprotein and red fluorescence due to rhodamine. Confocal laser scanning microscopy images (CLSM; a1 - d1) refers following red fluorescence due to rhodamine. Artifacts (asterisks). Magnification: 5× (FM) and 10× (CLSM). Scale bars: 200 μm (FM) and 100 μm (CLSM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Morphological findings in coronal pulp of the groups at 24 h and 14 days.

Parameter	Hemi-arches (n = 10)							
	CTRL		BLE		BLE + IBF-GEL		BLE + IBF-NEG	
	24h	14d	24h	14d	24h	14d	24h	14d
Vacuolar degeneration	2/ 10	4/ 10	4/ 10	0/ 10	6/ 10	0/ 10	8/ 10	0/ 10
Fibrosis	6/ 10	4/ 10	10/ 10	6/ 10	8/ 10	6/ 10	4/ 10	6/ 10
Odontoblastic layer loss	2/ 10	0/ 10	10/ 10	10/ 10	10/ 10	0/ 10	6/ 10	4/ 10
Odontoblastic layer disorganization	8/ 10	6/ 10	10/ 10	10/ 10	10/ 10	10/ 10	10/ 10	6/ 10
Tertiary dentin	0/ 10	0/ 10	2/ 10	0/ 10	2/ 10	8/ 10	2/ 10	6/ 10
Calcifications	4/ 10	4/ 10	0/ 10	6/ 10	0/ 10	8/ 10	0/ 10	8/ 10
Edema	4/ 10	2/ 10	10/ 10	6/ 10	10/ 10	4/ 10	10/ 10	0/ 10
Hyperemia	8/ 10	4/ 10	8/ 10	6/ 10	10/ 10	8/ 10	10/ 10	8/ 10
Necrosis	0/ 10	0/ 10	8/ 10	0/ 10	2/ 10	0/ 10	0/ 10	0/ 10

Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group.

dental pulp (Table 5; Table 6; Fig. 6A and a1). The morphological findings observed in the BLE and BLE + IBF-GEL groups were similar and included vacuolar degeneration, fibrosis, odontoblastic layer loss, edema, hyperemia, odontoblastic layer disorganization (occlusal and middle thirds), and necrosis (occlusal third) (Table 5; Fig. 6C, c1 and E, e1, respectively). The main morphological findings seen in the BLE + IBF-NEG group were vacuolar degeneration, odontoblastic layer disorganization in the pulp middle third, edema, and hyperemia. No necrosis was observed (Table 5; Fig. 6G and g1).

The highest inflammatory infiltrate scores in pulp tissue were observed in the BLE group, especially in the occlusal third (Table 6; Fig. 6C and c1). Most specimens that received the IBF-loaded hydrogel exhibited a mild inflammatory infiltrate (Table 6; Fig. 6E and e1). The BLE + IBF-NEG group showed a mild and moderate inflammatory infiltrate (Table 6; Fig. 6G and g1). No morphological alterations or

inflammatory infiltrate were observed in the cervical third (Table 6). Initial experiments were performed with the BLE+B-NEG150 group, which exhibited severe inflammatory activity in the coronal pulp after 24 h (Supplementary Fig. 3).

3.4.2. 14-Day morphological analysis

A reduction in edema and hyperemia was observed in all groups (Table 5; Fig. 6D-h1). Maintenance of odontoblast loss and disorganization of the odontoblastic layer were noted in the BLE and BLE+IBF-GEL groups (Table 5; Fig. 6D, d1 and F, f1, respectively). In the BLE+IBF-GEL and BLE+IBF-NEG groups, calcifications were formed and part of the pulp chamber was occupied by tertiary dentin (Table 5; Fig. 6F, f1 and H, h1, respectively). After 14 days, all groups showed a reduction in inflammatory infiltrate scores in the pulp. A mild inflammatory infiltrate in the occlusal third persisted only in the BLE+IBF-GEL group (Table 6; Fig. 6F and f1). A larger number of the morphological parameters and higher inflammatory infiltrate scores were observed after 24 h (for all treatment groups) compared to the 14-day period ($p < 0.05$) (Tables 5 and 6). Initial experiments were performed with the BLE+B-NEG150 group, which exhibited reduction in inflammatory activity after 14 days (Supplementary Fig. 3).

3.5. Immunohistochemical analysis

Representative images of IL-10, IL-1 β , SP, COX-2 and bradykinin immunostaining are shown in Fig. 7. The highest intensities of immunostaining for the inflammatory mediators were observed at the interval of 24 h in the coronal pulp of all groups. In the BLE group, a statistically significant difference between time points ($p < 0.05$) was found for IL-1 β , SP and bradykinin (Table 7). On the other hand, after 14 days, the BLE + IBF-GEL and BLE+IBF-NEG groups showed higher immunostaining intensities for IL-1 β ($p < 0.05$) and IL-10 ($p < 0.05$), respectively (Table 7 and Supplementary Table 1).

3.6. Comparison between groups

Statistically significant differences in the inflammatory infiltrate were observed between groups after 24 h ($p < 0.001$). The BLE group had a strong inflammatory infiltrate intensity compared to the CTRL and BLE+IBF-NEG groups ($p < 0.05$) (Table 6; Supplementary Table 2). 14 days after product application, there were no significant differences in

Table 6
Comparison of inflammatory infiltrate distribution according to coronal pulp third between groups at 24 h and 14 days.

Third	Score	Hemi-arches (n = 10)								p-value
		CTRL		BLE		BLE + IBF-GEL		BLE + IBF-NEG		
		24h	14d	24h	14d	24h	14d	24h	14d	
Occlusal	1	4/10	0/10	0/10	10/10	0/10	8/10	2/10	10/10	24h: $p < 0.001^c$; 14d: $p = 0.228$
	2	4/10	0/10	0/10	0/10	6/10	2/10	4/10	0/10	
	3	0/10	0/10	2/10	0/10	2/10	0/10	0/10	0/10	
	4	0/10	0/10	8/10	0/10	2/10	0/10	0/10	0/10	
Middle	1	0/10	8/10	0/10	0/10	0/10	0/10	0/10	0/10	
	2	2/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10	
	3	0/10	0/10	0/10	0/10	0/10	0/10	4/10	0/10	
	4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
Cervical	1	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
	2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
	3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
	4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
Median		2.0 ^a	1.0 ^b	4.0 ^a	1.0 ^b	2.0 ^a	1.0 ^b	2.0 ^a	1.0 ^b	

Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group.

The relative frequency of hemi-arches ranged from 0 to 10 for all groups.

Scores attributed to the intensity of the inflammatory infiltrate: 1 = absence of inflammatory cells or negligible number; 2 = mild inflammatory infiltrate (up to 25% of cells per field); 3 = moderate inflammatory infiltrate (between 25% and 75% of cells per field); 4 = severe inflammatory infiltrate (>75% cells per field) or necrosis.

^{a, b} Different letters in the row indicate a statistically significant difference between time points in the groups for Mann-Whitney test ($p < 0.05$), observed for the BLE, BLE + IBF-GEL and BLE + IBF-NEG groups.

^c Statistically significant difference between groups through Kruskal-Wallis test at 24 h and 14 days. df: degrees of freedom (df = 3).

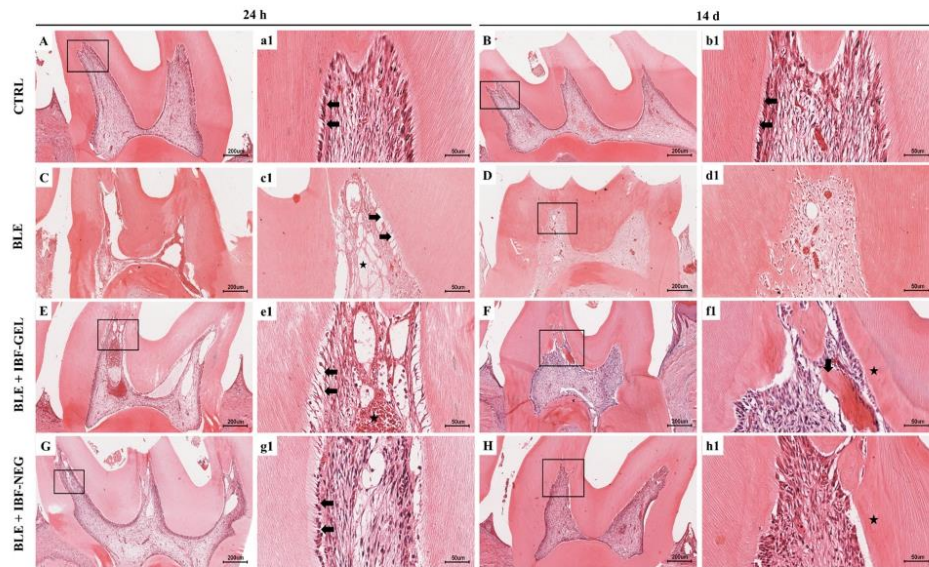


Fig. 6. Morphological analysis of the groups at 24 h and 14 days. Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group. 24 h: A) CTRL: scarce inflammatory infiltrate and loose connective pulp tissue with congested vessels and a1) organized odontoblastic layer (arrow); C) BLE: presence of necrosis and a severe inflammatory infiltrate and c1) edema (asterisk) and odontoblastic layer loss (arrow); E) BLE + IBF-GEL: edema and hyperemia with mild inflammatory infiltrate and e1) odontoblastic layer disorganization (arrow) and congested blood vessels (asterisk); G) BLE + IBF-NEG: coronal pulp with mild inflammatory infiltrate and edema areas and g1) odontoblastic layer organization (arrow). 14 days: B) CTRL: coronal pulp within normal limits and b1) organized odontoblasts (arrow); D) BLE: pulp showing extensive fibrosis and d1) odontoblastic layer loss in the occlusal and middle thirds; F) BLE + IBF-GEL: mild inflammatory infiltrate in dense connective tissue and f1) presence of tertiary dentin (asterisk) and nodular calcification in the pulp chamber (arrow); H) BLE + IBF-NEG: absence of inflammatory infiltrate and presence of some congested blood vessels and h1) tertiary dentin formation (asterisk). H&E staining. Scale bars: 200 μm (A–H); 50 μm (a1–h1).

the inflammatory infiltrate between groups ($p = 0.228$) (Table 6).

Immunohistochemical analysis showed statistically significant differences between all treatment groups after 24 h. On the other hand, after 14 days, significant differences between groups were only observed for IL10, IL-1 β , and COX-2 (Table 7).

Individual comparisons after 24 h showed higher IL-10 immunoreactivity in the BLE group, particularly when compared to the CTRL and BLE + IBF-NEG groups ($p < 0.05$). For IL-1 β , the BLE group exhibited the highest immunoreactivity compared to the CTRL and BLE+IBF-GEL groups ($p < 0.05$). Substance P was strongly immunoreactive in the BLE group compared to the CTRL and BLE+IBF-NEG groups ($p < 0.05$). Strong immunoreactivity of COX-2 was observed in the BLE group, mainly compared to the CTRL group ($p < 0.05$). Bradykinin showed strong immunostaining in the BLE group, especially between the BLE and CTRL groups and between the BLE+IBF-NEG and CTRL groups ($p < 0.05$). The BLE + IBF-GEL group showed moderate immunostaining for SP compared to the CTRL and BLE + IBF-NEG groups ($p < 0.05$) (Fig. 7; Table 7; Supplementary Tables 1 and 3).

4. Discussion

4.1. Preparation of thermosensitive IBF-loaded nanogel

The blank and IBF-loaded NEs were prepared using different surfactant ratios, resulting in an average droplet diameter < 600 nm [31]. The increase in surfactant ratio resulted in a larger droplet size, mainly due to the hydrophobic surfactant soybean phosphatidylcholine, which contributed to the expansion of the oil droplet surface [47]. However, the optimized blank NE formulations containing 1% SM showed a mean

droplet size of 98.5 nm, while that of the drug-loaded NE was 278.8 nm, reinforcing that IBF was successfully loaded into oil droplets [48,49].

The blank NEs and drug-loaded NEs exhibited different physical stability properties. Thus, the addition of P407 to the aqueous phase increased the physical stability because of its amphiphilic nature [36]. A clear decrease in droplet size was observed, even for the drug-loaded formulations. In this study, samples with concentrations less than 12.5% (w/w) remained as liquid dispersion at room temperature (25 $^{\circ}\text{C}$). Studies exploring these properties of P407 have found gelling formulations in the range of 15%–50% [50]. The tests using formulations containing 15%, 17.5%, and 20% (w/w) P407 demonstrated that 15% was sufficient to increase stability, to reduce droplet size, and to form NEs at room temperature. Kaplan et al. [51] also used poloxamer to induce NEs from NE formulations; however, the authors have employed a high-energy method to induce a droplet size of about 200 nm.

Poloxamer 407 is a nonionic copolymer and the observed zeta potential values contributed to stabilize the colloidal dispersions by steric hindrance and repulsion among droplets [47,52]. After 15% (w/w) P407 NE, it was possible to observe that both optimized drug-free and drug-loaded NE formulations remained with uniform droplet size distribution, corroborating its application as stabilizer in colloidal dispersions [41,53]. In addition, drug-loading in NEs decreased their stability and the presence of P407 was necessary for improving physical stability. The copolymer reduces Ostwald ripening phenomenon, which is characterized by the condensation of smaller droplets into a larger droplet and is one of the factors responsible for NE instability [54,55]. The IBF-NEG150 preserved as stable over 42 days and is quite satisfactory as nanometric droplets are important for topical administration since they

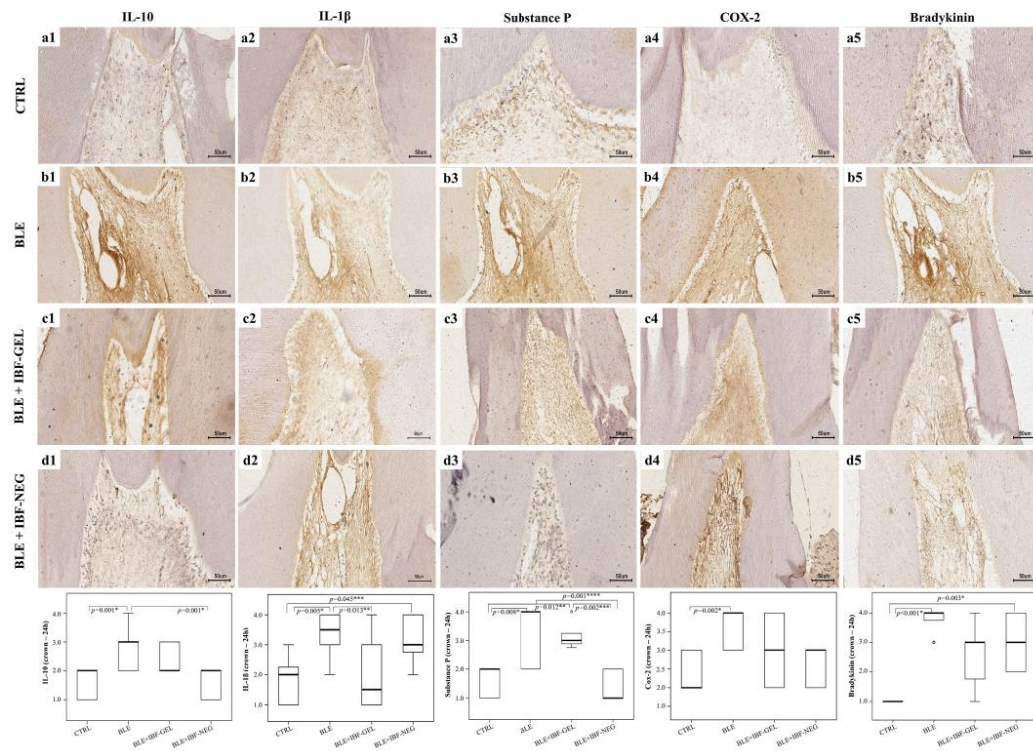


Fig. 7. Immunohistochemical analysis in coronal pulp of the groups at 24 h. Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group. a) CTRL: low immunoeexpression of a1) IL-10, a2) IL-1 β , a3) substance P and a4) COX-2, and absence of a5) bradykinin immunoeexpression. b) BLE group: moderate immunoeexpression of b1) IL-10 and b2) IL-1 β ; strong immunoeexpression of b3) substance P, b4) COX-2, and b5) bradykinin in the middle third. c) BLE + IBF-GEL group: low immunoeexpression of c1) IL-10 and c2) IL-1 β ; moderate immunoeexpression of c3) substance P, c4) COX-2, and c5) bradykinin in the occlusal third. d) BLE + IBF-NEG group: low immunoeexpression of d1) IL-10; moderate immunoeexpression of d2) IL-1 β in the occlusal third; absence of immunoeexpression of d3) substance P; moderate immunoeexpression of d4) COX-2 in the occlusal and middle thirds, and moderate expression of d5) bradykinin in the occlusal third. (** ***) $p < 0.05$: statistical significance by Dunn's post hoc test. Scale bar: 50 μ m (a1-d5).

contribute to an increase in surface area [56].

Finally, the *in vitro* IBF release studies revealed that both NE and NEG formulations were able to modify the drug release compared to the IBF solution. The ability of NE to increase bioadhesion and consequently to improve the residence time of the drug in the oral cavity and teeth should be also highlighted. This property is improved considerably when NEG is used because of its gelling ability when in contact with biological tissues. The experimental data reinforce that NEG was able to control the drug diffusion process [53,57]. This fact can be explained by the structure of the NEG; in this respect, the higher the poloxamer concentration, the higher the system's viscosity and consequently the slower the diffusion of the drug through the gel [58,59].

4.2. Tooth surface distribution and drug transport through dentinal tubules

Although some authors consider that the bleaching procedure is minimally invasive, with reversible effects [60], the use of bleaching agents on the tooth induces relevant morphological changes on the enamel surface, such as increased porosity, partial removal of the aprismatic layer, exposed enamel prisms, interprismatic substance loss, and significant reductions in the calcium/phosphorus ratio in enamel

and dentin after bleaching treatment [61,62]. These reductions resulted in a significant decrease of enamel microhardness [63] and induced tooth sensitivity [64].

It is also important highlight that these changes in enamel structure are related to the concentration and the duration of contact of the bleaching agent with the dental surface, facts that may be related to tooth sensitivity [65]. Among the hypotheses proposed to explain the mechanism of dentin hypersensitivity, the hydrodynamic theory remains the most widely accepted [66]. Thus, if the tubules are occluded in some part of their length, the hydraulic conductance will be reduced [67]. Previous studies demonstrate that dentin sensitivity can also be treated using nanocomposite systems, which obliterate the dentinal tubules and form a physical barrier on the surface [68,69]. In an *in vitro* study, a significant reduction in dentinal fluid flow rates was observed after treatment with an adhesive system. SEM images showed a significant correlation between the degree of occlusion of the dentinal tubules and the reduction in fluid flow [70].

In the present study, SEM images revealed the formation of an obliterating layer on the bleached tooth surface after 24 h in the groups receiving the IBF-loaded hydrogel and IBF-loaded nanogel. This fact corroborated their occlusion capacity and potential reduction of sensitivity. In addition, the drug remained homogeneously dispersed only in

Table 7
Immunostaining scores for inflammatory mediators in coronal pulp between groups at 24 h and 14 days.

Antibody	Scores	Hemi-arches (n = 10)								p-value
		CTRL		BLE		BLE + IBF-GEL		BLE + IBF-NEG		
		24h	14d	24h	14d	24h	14d	24h	14d	
IL-10	1	4/10	0/10	0/10	0/10	0/10	2/10	4/10	0/10	24h: $p < 0.001^c$ 14d: $p = 0.001^c$
	2	6/10	8/10	3/10	4/10	7/10	6/10	6/10	0/10	
	3	0/10	2/10	6/10	4/10	3/10	2/10	0/10	8/10	
	4	0/10	0/10	1/10	2/10	0/10	0/10	0/10	2/10	
	Median	2.0 ^a	2.0 ^b	3.0 ^a	3.0 ^a	2.0 ^a	2.0 ^a	2.0 ^a	3.0 ^b	
IL-1 β	1	4/10	2/10	0/10	0/10	5/10	0/10	0/10	0/10	24h: $p = 0.001^c$ 14d: $p = 0.002^c$
	2	4/10	8/10	1/10	6/10	2/10	2/10	2/10	6/10	
	3	2/10	0/10	4/10	4/10	2/10	5/10	5/10	2/10	
	4	0/10	0/10	5/10	0/10	1/10	3/10	3/10	2/10	
	Median	2.0 ^a	2.0 ^a	3.5 ^a	2.0 ^b	1.5 ^a	3.0 ^b	3.0 ^a	2.0 ^a	
SP	1	4/10	6/10	0/10	2/10	0/10	2/10	6/10	0/10	24h: $p < 0.001^c$ 14d: $p = 0.105$
	2	6/10	2/10	4/10	6/10	2/10	2/10	4/10	8/10	
	3	0/10	2/10	0/10	2/10	6/10	6/10	0/10	2/10	
	4	0/10	0/10	6/10	0/10	2/10	0/10	0/10	0/10	
	Median	2.0 ^a	1.0 ^a	4.0 ^a	2.0 ^b	3.0 ^a	3.0 ^a	1.0 ^a	2.0 ^b	
COX-2	1	0/10	8/10	0/10	0/10	0/10	4/10	0/10	0/10	24h: $p = 0.003^c$ 14d: $p = 0.003^c$
	2	6/10	0/10	0/10	4/10	4/10	0/10	3/10	2/10	
	3	4/10	2/10	4/10	2/10	3/10	6/10	7/10	7/10	
	4	0/10	0/10	6/10	4/10	3/10	0/10	0/10	1/10	
	Median	2.0 ^a	1.0 ^b	4.0 ^a	3.0 ^a	3.0 ^a	3.0 ^a	3.0 ^a	3.0 ^a	
Bradykinin	1	10/10	0/10	0/10	0/10	2/10	0/10	0/10	1/10	24h: $p < 0.001^c$ 14d: $p = 0.325$
	2	0/10	8/10	0/10	6/10	1/10	4/10	4/10	5/10	
	3	0/10	2/10	2/10	2/10	6/10	4/10	2/10	3/10	
	4	0/10	0/10	8/10	2/10	1/10	2/10	4/10	1/10	
	Median	1.0 ^a	2.0 ^b	4.0 ^a	2.0 ^b	3.0 ^a	3.0 ^a	3.0 ^a	2.0 ^a	

Four groups of five animals in each group, totaling 10 hemi-arches analyzed per group.

The relative frequency of hemi-arches ranged from 0 to 10 for all groups.

Immunostaining scores: 1 = absence of immunostaining (absence of staining in the extracellular matrix and complete absence of immunoreactive cells); 2 = low immunoreactivity (weak staining of the extracellular matrix and approximately one quarter of immunoreactive cells); 3 = moderate immunoreactivity (moderate staining of the extracellular matrix and approximately one half of immunoreactive cells); 4 = strong immunoreactivity (strong staining of the extracellular matrix and approximately three quarters of immunoreactive cells).

^{a, b} Different letters in the rows indicate statistically significant differences for Mann-Whitney test ($p < 0.05$), between time points in the groups of each antibody analyzed. IL-10: CTRL and BLE + IBF-NEG; IL-1 β : BLE and BLE + IBF-GEL; SP: BLE and BLE + IBF-NEG; COX-2: CTRL; Bradykinin: CTRL and BLE.

^c Statistically significant difference between groups through Kruskal-Wallis test at 24 h and 14 days. df: degrees of freedom (df = 3).

the BLE + IBF-NEG group, demonstrating superior ability for nanogel formulation. Methods aimed at obliterating the dentinal tubules may therefore be beneficial in the treatment of dentin sensitivity and SEM analysis is an appropriate method for visualizing particles on the tooth surface [71].

Furthermore, oil nanodroplets dispersed in the aqueous colloidal gel carrier seem to be necessary for induces the formation of a more uniform layer on the enamel surface when compared to the conventional IBF-loaded hydrogel. In the BLE+IBF-GEL group, the supposed residual presence of IBF precipitate reinforces this fact, suggesting a possible low drug concentration in the dental pulp. This hypothesis was better investigated on the tooth structure by using fluorescence and CSLM images [72,73]. In the current approach, fluorescence and CSLM images corroborates our hypothesis. In agreement with our results, an *in vitro* study using CSLM reported that rhodamine B penetrated the tooth structure when it was applied to the external tooth surface and reached the pulp cavity, suggesting that bleaching agents possibly exhibit similar penetration pathways [74]. Within this context, Cavalli et al. [75], who added rhodamine to H₂O₂ and traced the bleaching agent using CSLM, observed diffusion of H₂O₂ into the pulp chamber.

Thus, the IBF-loaded hydrogel and IBF-loaded nanogel associated with rhodamine B were visualized on the enamel surface and in the dentinal tubules, reaching the dental pulp, with marked fluorescence emissions in the rhodamine B range. However, the IBF-loaded hydrogel delivered greater rhodamine amount on the tooth surface, while the IBF-loaded nanogel demonstrated a greater rhodamine amount distributed into dentinal tubules to the dental pulp. This fact can be explained due to the diffusion of rhodamine-loaded nanodroplets through the micrometric channels (>1000 nm) identified on tooth surface connected to the

dentinal tubules and finally to the dental pulp. The capacity of the NE as smart material able for specific drug delivery is well established in the literature [76]. This ability for topical dental application is demonstrated for the first time in the present approach.

4.3. Morphological and immunohistochemical studies

The pulp tissues of the animals submitted to bleaching and treated with topical administration of IBF showed less damage than that of the BLE group that did not receive the anti-inflammatory agent. The morphological findings corroborated the hypothesis that thermosensitive IBF-loaded nanogel reduced the inflammation caused by the bleaching agent and exhibited the lowest inflammatory response of the pulp tissue, rejecting the null hypothesis. As expected, 24 h after bleaching, the BLE group showed the highest scores of inflammatory infiltration as well as areas of necrosis, corroborating previous studies [18,46,77]. The reduction of inflammatory infiltrate scores and the absence of necrosis were observed when the IBF-loaded nanogel was administered topically before and after the bleaching treatment. The conventional IBF-loaded hydrogel was unable to avoid necrosis, demonstrating the superior performance for nanogel sample.

Pulp inflammation causes edema, which increases fluid accumulation in the dentinal tubules and consequent painful sensation [78]. In this study, edema was present in all groups, especially 24 h after bleaching. A considerable decrease in edema was observed in the groups that received the IBF-loaded nanogel 14 days after treatment as a result of the reduction in the inflammatory infiltrate, a fact that may contribute to the reduction of sensitivity. There are reports of reduced tooth sensitivity in patients over longer intervals after completion of the

bleaching treatment [79]. This finding can be explained by the fact that the products of different classes of oxidative reactions can be considered to represent damage-associated molecular patterns (DAMPs) and, consequently, are targets of multiple mechanisms of the innate immunity [80]. Furthermore, the BLE+IBF-GEL and BLE+IBF-NEG groups showed the formation of tertiary dentin occupying part of the pulp chamber 14 days after completion of the bleaching treatment. This fact supports with the “protective factor” effect when anti-inflammatory agents are successfully applied. It is interesting reinforces that similar studies only observed this fact 30 days after bleaching procedure [23,81, 82]. These data suggest the interesting properties of IBF formulations as potential accelerator of the formation of tertiary dentin in bleached teeth.

The collagen fiber deposition was observed 24 h after tooth bleaching in the BLE group, while in the BLE+IBF-NEG group this fact was observed 14 days after the procedure. This limitation suggests further investigation. Fibrosis is the pulp’s reactional response to the aggression promoted by bleaching leading to the release of inflammatory mediators and a consequent decrease in the reparative capacity of the pulp [81, 83].

Although similar patterns were observed for the BLE+B-NEG150 group related to biadhesion and penetration into the tooth surface found by rhodamine, this formulation appears to have no anti-inflammatory effect in the pulp tissue, demonstrating similar patterns observed in the BLE group. Therefore, the IBF presence in the nanogel is essential to reduce the inflammatory effect promoted by the bleaching procedure [49].

Immunohistochemical analysis indicated moderate IL-10 immunostaining in the BLE group after 24 h. On the other hand, higher IL-10 immunostaining was observed in the BLE+IBF-NEG group after 14 days. This cytokine is the main inhibitor of macrophage activity and proinflammatory cytokine synthesis [20]. In addition, the topical use of conventional IBF-loaded hydrogel reduced IL-1 β levels at 24 h. IL-1 β promotes vasodilation and induces the expression of prostaglandins [84]. As reported in other studies, higher levels of IL-1 β were observed after exposure to H₂O₂ [15,16]. The assessment of SP levels provides a more accurate information on pain relief methods [85,86]. IBF-loaded nanogel was the only formulation able to induce considerable reduction in expression of this neuropeptide mediator. Some authors found higher expression of SP in teeth exposed to H₂O₂ [17,18]; however, the combination with an anti-inflammatory agent reduced SP immunostaining 24 h after treatment [18], similarly to our study. This result demonstrates its efficacy and incredible potential as topical analgesic to minimize the painful effects of bleaching. For the first time, COX-2 and bradykinin levels were evaluated for *in vivo* tooth bleaching studies. Higher levels of COX-2 and bradykinin were observed in the BLE group with no difference when compared to IBF formulations. This fact can be explained due to the time necessary to IBF reaches minimal effective concentration in the dental pulp [87,88] or due to the topical administration route, considered a challenge for drug delivery differences.

The concept proof of the nanosystems with anti-inflammatory action for topical application on the tooth surface was successfully achieved. The presented data and discussed results are promising to continue with clinical studies. Certainly, advanced studies need to be performed to accomplish specific demands of regulatory agencies such as the *Food Drug Administration*, as storage stability, for example. In addition, different drugs, or interesting biomolecules to be used in dentistry can also be tested.

5. Conclusions

In this study, a stable IBF-loaded nanogel with nanometric properties, capable of carrying satisfactory amounts of the drug for topical administration, was prepared and various parameters were analyzed. In conclusion, the drug release profile makes this nanogel a potentially promising alternative vehicle for the topical application of IBF and other

drugs in dentistry. Our results indicate the penetration capacity of the IBF-loaded nanogel in the tooth, minimizing the inflammatory damage caused by bleaching in the pulp tissue of Wistar rats.

Credit author statement

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Declaration of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

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6. CONCLUSÕES

A utilização de ratos como modelo experimental apresenta vantagens como facilidade de manipulação, reprodução e previsibilidade de resultados. Estes animais apresentam as proporções das estruturas dentárias, assim como as características histológicas e fisiológicas dos molares similares às dos humanos, com reações biológicas comparáveis, podendo ser utilizados como modelo para prever resultados de procedimentos realizados em incisivos inferiores humanos.

Deste modo, pela primeira vez em um estudo *in vivo*, foram avaliadas as alterações no tecido pulpar de ratos Wistar submetido ao clareamento associado à administração tópica de um nanogel termorresponsivo carregado com ibuprofeno. Vários parâmetros desta formulação inovadora com propriedades nanométricas foram analisados e foi observada a sua capacidade de penetração e transporte de quantidades satisfatórias do fármaco para o tecido pulpar minimizando o dano inflamatório causado pelo procedimento clareador. Assim, o perfil de liberação torna este nanogel um veículo promissor para a aplicação tópica do ibuprofeno na odontologia.

Portanto, a prova conceito dos nanossistemas com ação anti-inflamatória para aplicação tópica na superfície dentária foi alcançada com sucesso. Os dados apresentados e os resultados discutidos são promissores para dar continuidade a estudos clínicos. Certamente, estudos mais avançados precisam ser realizados a fim de atender a demandas específicas de agências reguladoras como a *Food Drug Administration*, dentre elas a estabilidade de armazenamento. Além disso, outros fármacos ou biomoléculas de uso odontológico também poderão ser testados nesse sistema.

7. COMENTÁRIOS, CRÍTICAS E SUGESTÕES

O caráter multiprofissional do Programa de Pós-graduação em Ciências da Saúde, bem como a realização deste estudo, foram de extrema importância para a ampliação dos meus conhecimentos na área da pesquisa científica, que além de contribuir para meu desenvolvimento intelectual, foram valiosos para realização de alterações metodológicas relevantes no anteprojeto inicial que permitiram a continuidade deste estudo. O contato com profissionais, professores e colegas de turma, das mais diversas áreas da saúde, permitiram importantes trocas de experiências dentro e fora das dimensões da pesquisa, sendo enriquecedor para o aprimoramento individual e coletivo.

Diversas limitações, principalmente relacionadas à infra-estrutura, bem como dificuldades metodológicas iniciais inerentes a este estudo foram encontradas. Para superá-las, o investimento pessoal, a colaboração de parceiros, a perseverança e a expectativa em resultados positivos foram fundamentais para superar os obstáculos e valorizar ainda mais cada conquista.

A realização de diferentes análises em diversas áreas do conhecimento contribuiu para a multidisciplinaridade e engrandecimento deste estudo. A sua originalidade, a partir do desenvolvimento de uma nova modalidade terapêutica para redução da inflamação pulpar ocasionada pelo clareamento dental, justifica a sua realização e contribui sobremaneira como ponto de partida para que mais estudos sejam realizados nesta área, a fim de que sejam utilizadas estratégias que possibilitem a redução da sensibilidade referida pelos pacientes e os permita realizar o tratamento clareador de forma indolor e mais segura.

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9. APÊNDICE

9.1 Material Suplementar

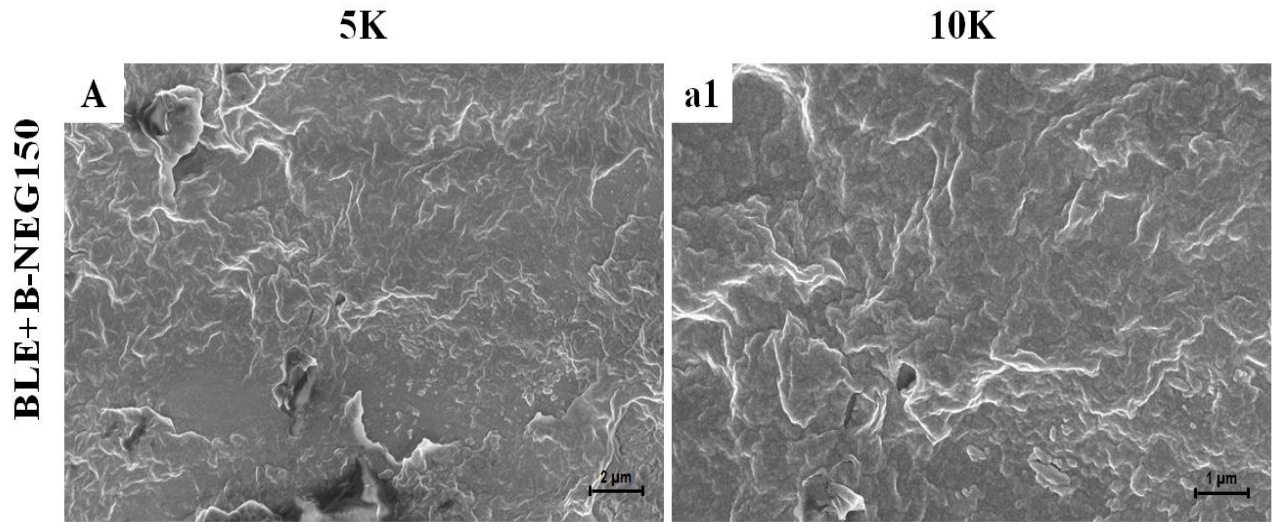


Figura suplementar 1. Imagens de microscopia eletrônica de varredura (MEV) de molares de ratos Wistar (grupo BLE+B-NEG150). (A - a1) Formação de uma camada uniforme na superfície do dente clareado após 24 horas. Magnificações: 5000x (A) e 10000x (a1).

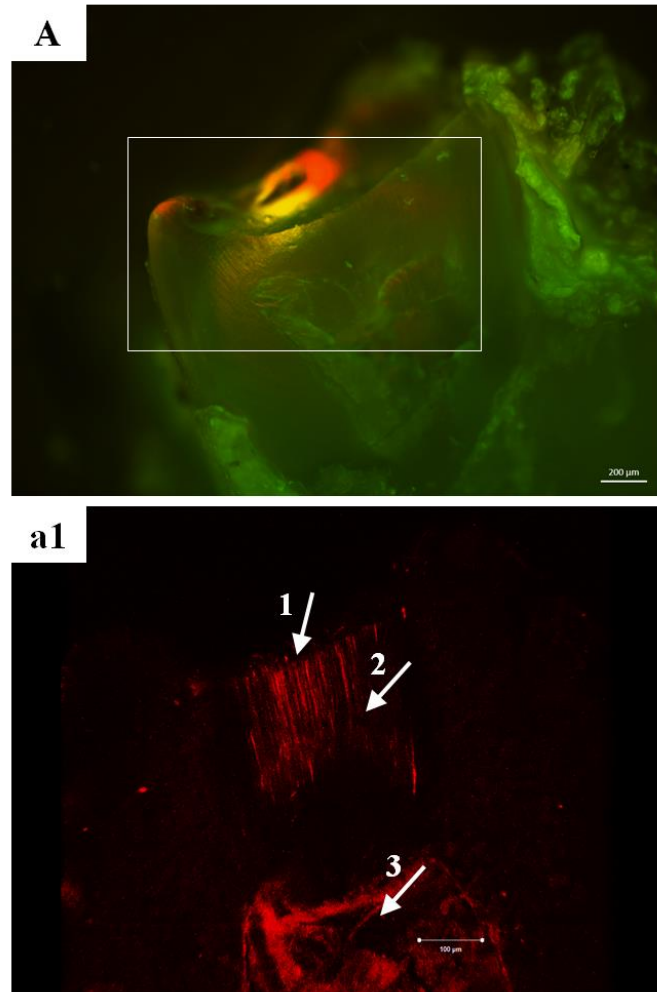
BLE+B-NEG150

Figura suplementar 2. Estudo de transporte do nanogel branco (B-NEG150) através dos túbulos dentinários, após 24 horas, através da microscopia de fluorescência e microscopia confocal de varredura a laser. Imagem representativa da microscopia de fluorescência (MF; A) da amostra do grupo BLE+B-NEG150: fluorescência verde devido à fosfoproteína dentinária e fluorescência vermelha devido à rodamina B. Imagem representativa da microscopia confocal de varredura a laser (MCVL; a1): fluorescência vermelha devido à presença da rodamina B. Magnificação: 5x (MF) e 10x (MCVL). Barras de escala: 200 μm (MF) e 100 μm (MCVL).

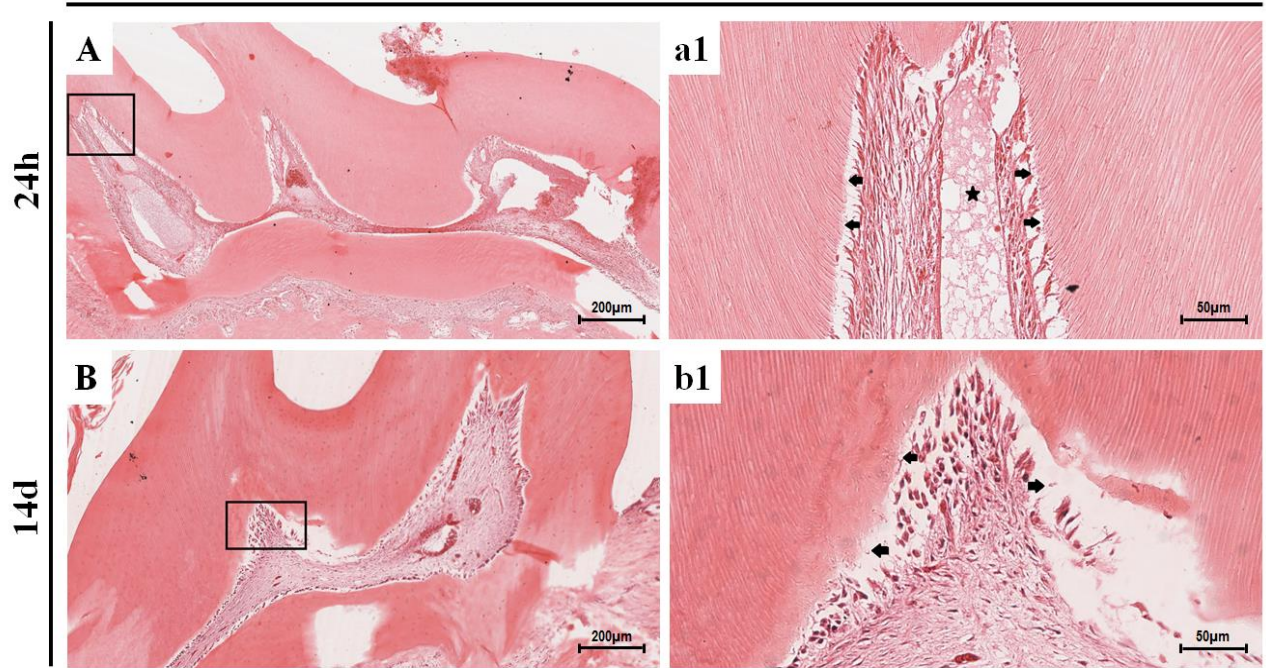
BLE+B-NEG150

Figura suplementar 3. Análise morfológica do grupo BLE+B-NEG150 em 24 horas e 14 dias. 24 horas: A) necrose, infiltrado inflamatório severo, edema, hiperemia e a1) desorganização da camada odontoblástica (seta) e edema (asterisco). 14 dias: B) hiperemia e fibrose com leve infiltrado inflamatório e b1) desorganização da camada odontoblástica (seta). Coloração H&E. Barras de escala: 200 µm (A-B); 50 µm (a1-b1).

Tabela Suplementar 1. Comparações individuais dos escores dos mediadores inflamatórios entre os grupos.

Anticorpo	Polpa coronária	24 h	14 d
		<i>p-value</i>	<i>p-value</i>
	Comparação entre os grupos		
IL-10	CTRL vs. BLE+IBF-GEL	0.462	1.000
	CTRL vs. BLE+IBF-NEG	<0.001*	0.007*
	CTRL vs. BLE	0.009*	0.402
	BLE+IBF-NEG vs. BLE+IBF-GEL	0.031*	0.001*
	BLE+IBF-GEL vs. BLE	0.946	0.147
	BLE+IBF-NEG vs. BLE	1.000	0.941
IL-1 β	CTRL vs. BLE+IBF-GEL	1.000	0.001*
	CTRL vs. BLE+IBF-NEG	0.045*	0.146
	CTRL vs. BLE	0.005*	0.336
	BLE+IBF-NEG vs. BLE+IBF-GEL	0.094	0.696
	BLE+IBF-GEL vs. BLE	0.013*	0.336
	BLE+IBF-NEG vs. BLE	1.000	1.000
COX-2	CTRL vs. IBF-GEL	0.847	0.580
	CTRL vs. IBF-NEG	1.000	0.012*
	CTRL vs. BLE	0.002*	0.006*
	BLE+IBF-NEG vs. BLE+IBF-GEL	1.000	0.937
	BLE+IBF-GEL vs. BLE	0.209	0.631
	BLE+IBF-NEG vs. BLE	0.052	1.000
Substância P	CTRL vs. BLE+IBF-GEL	0.012*	
	CTRL vs. BLE+IBF-NEG	1.000	
	CTRL vs. BLE	0.008*	
	BLE+IBF-NEG vs. BLE+IBF-GEL	0.002*	
	BLE+IBF-GEL vs. BLE	1.000	
	BLE+IBF-NEG vs. BLE	0.001*	
Bradicinina	CTRL vs. BLE+IBF-GEL	0.051	
	CTRL vs. BLE+IBF-NEG	0.003*	
	CTRL vs. BLE	<0.001*	
	BLE+IBF-NEG vs. BLE+IBF-GEL	1.000	
	BLE+IBF-GEL vs. BLE	0.101	
	BLE+IBF-NEG vs. BLE	0.780	

* Diferença estatisticamente significativa realizada através do pós teste de Dunn.

Tabela Suplementar 2. Comparação individual dos escores entre os grupos.

Comparação dos grupos	<i>p-value</i>
24 horas (polpa coronária)	
CTRL vs. BLE+IBF-NEG	0.962
CTRL vs. BLE+IBF-GEL	0.164
CTRL vs. BLE	<0.001*
BLE+IBF-NEG vs. BLE+IBF-GEL	1.000
BLE+IBF-NEG vs. BLE	0.005*
BLE+IBF-GEL vs. BLE	0.069

Pós teste de Dunn; graus de liberdade = 3.

Tabela Suplementar 3. Distribuição da imunexpressão dos mediadores inflamatórios de acordo com o terço da polpa coronária nos grupos em 24 horas e 14 dias.

		CTRL									
Terço	Escore	IL10		IL-1β		Substância P		COX-2		Bradicinina	
		24h	14d	24h	14d	24h	14d	24h	14d	24h	14d
Oclusal	1	0/10	0/10	4/10	2/10	4/10	6/10	0/10	8/10	10/10	0/10
	2	2/10	8/10	4/10	6/10	6/10	2/10	4/10	0/10	0/10	4/10
	3	0/10	0/10	2/10	0/10	0/10	2/10	2/10	2/10	0/10	2/10
	4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Médio	1	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	2	2/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	4/10
	3	0/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Cervical	1	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	2	2/10	0/10	0/10	2/10	0/10	0/10	2/10	0/10	0/10	0/10
	3	0/10	0/10	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10
	4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		BLE									
Terço	Escore	IL10		IL-1β		Substância P		COX-2		Bradicinina	
		24h	14d	24h	14d	24h	14d	24h	14d	24h	14d
Oclusal	1	0/10	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10	0/10
	2	3/10	2/10	1/10	2/10	4/10	4/10	0/10	4/10	0/10	2/10
	3	4/10	2/10	3/10	2/10	0/10	2/10	2/10	0/10	0/10	0/10
	4	1/10	0/10	4/10	0/10	4/10	0/10	6/10	4/10	0/10	0/10
Médio	1	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	2	0/10	2/10	0/10	4/10	0/10	0/10	0/10	0/10	0/10	0/10
	3	2/10	0/10	0/10	2/10	0/10	0/10	0/10	2/10	0/10	2/10
	4	0/10	2/10	0/10	0/10	2/10	0/10	0/10	0/10	8/10	2/10
Cervical	1	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	2	0/10	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10	4/10
	3	0/10	2/10	1/10	0/10	0/10	0/10	2/10	0/10	2/10	0/10
	4	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		BLE+IBF-GEL									
Terço	Escore	IL10		IL-1β		Substância P		COX-2		Bradicinina	
		24h	14d	24h	14d	24h	14d	24h	14d	24h	14d

10. ANEXO


Universidade Federal do Rio Grande do Norte
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

PROTOCOLO N.º 037/2016

 Professor/Pesquisador: *HEBEL CAVALCANTI GALVÃO*

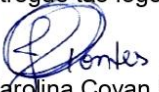
Natal (RN), 06 de setembro de 2016.

CERTIFICADO

Certificamos que a proposta intitulada "**Avaliação do efeito protetor de substâncias anti-inflamatórias e analgésicas administradas tópica e sistemicamente sobre as alterações pulpares promovidas pelo clareamento dentário de consultório: um estudo em ratos Wistar**" com o nº 037/2016, sob a responsabilidade de **Hebel Cavalcanti Galvão** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE – CEUA/UFRN, em reunião de 03/08/2016.

Finalidade	(X) Ensino () Pesquisa Científica
Vigência da Autorização	FEVEREIRO DE 2019
Número de Animais	90
Espécie/Linhagem/Raça	<i>Rattus norvegicus</i> Wistar
Peso/Idade	300g / 2 meses
Sexo	Machos
Origem	Biotério Depto Biofísica e Farmacologia - UFRN
Manutenção/Experimentação	Biotério Depto Biofísica e Farmacologia - UFRN

Informamos ainda que, segundo o Cap. 2, Art. 13 do Regimento, é função do professor/pesquisador responsável pelo projeto a elaboração de relatório de acompanhamento que deverá ser entregue tão logo a pesquisa for concluída.


 Josy Carolina Covan Pontes
 Coordenadora da CEUA



Capacitação no Uso e Manejo de Animais de Laboratório

Declaração de conclusão de curso

Declaramos para os devidos fins que

Samara Kelly Silva Chaves Fernandes Moura

participou do curso de "Capacitação no Uso e Manejo de Animais de Laboratório" em formato de ensino a distância, coordenado pela Central de Bioterismo do Instituto de Ciências Biomédicas em parceria com a Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e realizado pela REBIOTERIO- CNPq.

São Paulo, 24 julho 2019

Central de Bioterismo
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