JOHNNY CARVALHO DA SILVA

AVALIAÇÃO DA RESPOSTA IMUNE EM CÉLULAS PULPARES SENESCENTES E NÃO SENESCENTES E A BUSCA POR PEPTÍDEOS SENOTERAPÊUTICOS

BRASÍLIA, 2025.

UNIVERSIDADE DE BRASÍLIA

FACULDADE DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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Dissertação apresentada como requisito parcial para a obtenção do Título de Mestre em Ciências da Saúde pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Brasília.

Orientadora: Profa. Dra. Taia Maria Berto Rezende

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Aprovado em 24 de fevereiro de 2025

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Dedico este trabalho aos estudantes de pós-graduação, que enfrentam desafios com coragem e persistência, acreditando na transformação que o conhecimento proporciona.

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“Somos o que fazemos repetidamente. A excelência, então, não é um ato, mas um hábito.”

Aristóteles

RESUMO

A senescência celular é caracterizada pela interrupção irreversível do ciclo celular, acompanhada de alterações morfológicas, metabólicas, lisossomais e nos fatores de secreção. Embora tenha evoluído inicialmente como um mecanismo para limitar a proliferação de células envelhecidas ou danificadas, a senescência contribui para o envelhecimento, resultando em doenças relacionadas à idade e na diminuição das funções celulares. Essas alterações também impactam as células da polpa dentária, prejudicando suas funções defensivas e sensoriais, podendo comprometer o sucesso de tratamentos endodônticos conservadores. Este trabalho foi dividido em 3 capítulos. No capítulo 1, buscamos explorar de maneira aprofundada a senescência no contexto da odontologia, com foco no complexo dentino-pulpar, através de uma revisão de literatura com elementos e análises bibliométricas. A pesquisa também explora a literatura científica para identificar produtos odontológicos que induzem senescência celular. Além disso, o estudo discute potenciais estratégias para mitigar os efeitos da senescência. Nosso estudo conclui que, apesar do aumento nas pesquisas sobre senescência no complexo dentino-pulpar, o tema ainda exige maior exploração. É crucial acelerar e aprofundar estudos para identificar produtos que induzam ou mitiguem a senescência, traduzindo esse conhecimento em soluções clínicas eficazes. O capítulo 2 teve como objetivo avaliar a morfologia, migração, proliferação, viabilidade celular e a resposta imune de células da polpa dentária humana em senescência e sob estimulação inflamatória. Inicialmente, a senescência foi induzida com doxorrubicina e confirmada por coloração de β-galactosidase. Para simular uma resposta inflamatória *in vitro*, utilizou-se lipopolissacarídeo (LPS) e interferon-gama (IFN-γ). As alterações morfológicas foram analisadas por microscopia eletrônica de varredura. A proliferação e a viabilidade celular foram avaliadas por exclusão com azul de tripano, enquanto a capacidade de migração celular foi determinada pelo método *scratch*. A resposta imune foi investigada por meio da expressão de genes de citocinas pró-inflamatórias, como *indoleamina 2,3-dioxigenase (IDO), fator de necrose tumoral alfa (TNF-α)* e *interleucina 6 (IL-6)*, e citocinas anti-inflamatórias, como *IL- 10* e *fator de crescimento transformador beta 1 (TGF-β1)*, utilizando ensaio de qPCR. Os resultados demonstraram que células senescentes apresentaram maior tamanho, menor número de prolongamentos, redução na migração, proliferação e viabilidade,

além de aumento na expressão de *IDO, TNF-α* e *IL-6*. A expressão de *TGF-β1* foi reduzida no grupo tratado com LPS, enquanto a expressão de *IL-10* apresentou aumento sem significância estatística. O capítulo 3 avaliou o potencial senoterapêutico de uma biblioteca de 15 peptídeos desenhados por inteligência artificial. Para isso, uma biblioteca de peptídeos, sintetizados após design *in silico*, foi avaliada antes e após a indução da senescência com doxorrubicina. A triagem dos peptídeos foi realizada por coloração de β-galactosidase, enquanto a viabilidade celular foi analisada pelo ensaio MTT, e a produção de óxido nítrico (NO) foi mensurada pelo método de Griess. Dentre os peptídeos testados, dois peptídeos apresentaram bons potenciais, sendo eles C3 e C10, por apresentarem boa viabilidade celular mesmo em altas concentrações, redução da marcação por β-galactosidase e níveis de produção de nitrito próximos a condição basal. De modo geral, os resultados do nosso estudo indicam a presença de inflamação exacerbada associada à imunossupressão, comprometendo o potencial reparador das células pulpares senescentes, além de redução da migração e proliferação celular. Nesse contexto, a senescência celular pode influenciar negativamente o prognóstico de tratamentos endodônticos conservadores, prejudicando funções celulares essenciais para sua eficácia. Em adição, também foram observados que vários produtos utilizados no contexto do complexo dentino pulpar apresentam potencial para indução de senescência pulpar. Desta forma, a busca por novas drogas com potencial senoterapêutico surge, como uma alternativa promissora para mitigar os efeitos da senescência na polpa dentária. Neste sentido, os peptídeos mais promissores nesta análise inicial serão avaliados em testes futuros.

Palavras-chave: Senescência; Envelhecimento; Polpa dentária; Peptídeos.

ABSTRACT

Cellular senescence is characterized by the irreversible arrest of the cell cycle, accompanied by morphological, metabolic, lysosomal, and secretory factor alterations. Although it initially evolved as a mechanism to limit the proliferation of aged or damaged cells, senescence contributes to aging, resulting in age-related diseases and the decline of cellular functions. These changes also affect dental pulp cells, impairing their defensive and sensory functions and potentially compromising the success of conservative endodontic treatments. This study was divided into three chapters. Chapter 1 aims to explore senescence in dentistry, focusing on the dentin-pulp complex through a literature review with bibliometric analysis. The research also investigates the scientific literature to identify dental products that induce cellular senescence. Furthermore, the study discusses potential strategies to mitigate the effects of senescence. Our study concludes that, despite increasing research on senescence in the dentin-pulp complex, the topic requires further exploration. Accelerating and deepening studies to identify products that induce or mitigate senescence and translating this knowledge into effective clinical solutions is crucial. Chapter 2 aimed to evaluate the morphology, migration, proliferation, viability, and immune response of human dental pulp cells in senescence and under inflammatory stimulation. Initially, senescence was induced using doxorubicin and confirmed by β-galactosidase staining. To simulate an *in vitro* inflammatory response, lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) were used. Morphological changes were analyzed by scanning electron microscopy. Cell proliferation and viability were assessed by trypan blue exclusion, while migration capacity was determined using the scratch assay. The immune response was investigated by assessing the expression of pro-inflammatory cytokine genes, such as *indoleamine 2,3-dioxygenase (IDO)*, *tumor necrosis factor-alpha (TNF-α)*, and *interleukin-6 (IL-6)*, and anti-inflammatory cytokines, such as *IL-10* and *transforming growth factor beta 1 (TGF-β1)*, using qPCR. Results demonstrated that senescent cells showed increased size, fewer extensions, reduced migration, proliferation, and viability, along with increased expression of *IDO, TNF-α*, and *IL-6*. *TGF-β1* expression was reduced in the LPS-treated group, while *IL-10* expression increased without statistical significance. Chapter 3 evaluated the senotherapeutic potential of a library of 15 peptides designed using artificial intelligence. This peptide library, synthesized after *in silico* design, was

tested before and after senescence induction with doxorubicin. The screening of peptides was performed using β-galactosidase staining, while cell viability was assessed using the MTT assay, and nitric oxide (NO) production was measured using the Griess method. Among the peptides tested, two peptides, C3 and C10, showed good potential, as they maintained high cell viability even at high concentrations, reduced β-galactosidase staining, and nitrite production levels close to basal conditions. Overall, the results of our study indicate the presence of exacerbated inflammation associated with immunosuppression, compromising the reparative potential of senescent dental pulp cells, along with reduced migration and proliferation. In this context, cellular senescence may negatively influence the prognosis of conservative endodontic treatments by impairing essential cellular functions. Additionally, several products used in the context of the dentin-pulp complex were observed to have the potential to induce pulp senescence. Thus, the search for new drugs with senotherapeutic potential emerges as a promising alternative to mitigate the effects of senescence in the dental pulp. In this regard, the most promising peptides from this initial analysis will be evaluated in future tests.
Keywords: Senescence; Aging; Dental Pulp; Peptides.

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CAPÍTULO 1.

between 2004-2008. C: publications between 2009-2013. D: publications between 2014-2018. E: 2019-2024. A minimum of 4 studies was established for the selection of keywords. The most recurring keywords are represented by larger points and in enlarged font, while the less frequent ones are associated with smaller points and presented in reduced font. The lines connecting the points illustrate the relationships and the shared use of these words in the studies considered. Created by VOSViewer.

CAPÍTULO 2

Figure 1. Human dental pulp cells after β-galactosidase staining assay and their viability under different conditions. (A) Schematic diagram of induced senescence protocol and its confirmation by β-galactosidase staining assay, along with the in vitro immune-inflammatory system model. Non-senescent (B) and senescent (C) cells. Scale bar: 500 μm. Number of senescent human dental pulp cells after β-galactosidase staining (D). ***$p < 0.0002$, by Student's t-test. Viability of non-senescent and senescent primary pulp cells in untreated (UT) culture (E), in LPS-stimulated culture (F), in LPS plus IFN-γ-stimulated culture (G), and comparative analysis between all assay groups (H). *$p < 0.05$, by one-way ANOVA test.

Figure 2. Morphological analysis of senescent and non-senescent pulp cells under different conditions. Dental pulp cells were photographed using SEM at magnifications of 1500x, 3000x, and 5000x, as shown in the columns. Each row represents a different cell condition: untreated, treated with LPS, and treated with LPS plus IFN-γ (A). The quantitative analysis of the images obtained by SEM compared the senescent group to the non-senescent group under different treatment conditions, considering the number of cell extensions and cell size. Analyses for each treatment condition are presented for the untreated group (UT) (B, E), for the group in the presence of LPS (C, F), and for the group stimulated by LPS plus IFN-γ (D, G). ****$p < 0.0001$, by one-way ANOVA test.

Figure 3. Migration and proliferation of senescent and non-senescent pulp cells under different conditions. Representative images of the migration: Non-senescent: untreated (UT) (A, D, G), in the presence of LPS (B, E, H), stimulated by LPS plus IFN-γ (C, F, I); Senescent: UT (J, M, P), in the presence of LPS (K, N, Q), stimulated by

LPS plus IFN-γ (L, O, R). Time points: 0h (A, B, C, J, K, L), 24h (D, E, F, M, N, O), and 48h (G, H, I, P, Q, R). Yellow dots denote the presence of cells within the wound. Graphical representation of the data observed in the migration and proliferation assays: UT (S, W), groups in the presence of LPS (U, Y), groups stimulated by LPS plus IFN-γ (T, X), and comparative analysis between all assay groups (V, Z). **p <0.0013 and ****p <0.0001, by one-way ANOVA test.

Figure 4. Gene expression of inflammatory and anti-inflammatory mediators by senescent and non-senescent pulp cells in different experimental conditions. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* was used as constitutive gene. *IDO* expression (A, B, C); *TNF-α* expression (D, E, F); *IL-6* expression (G, H, I); *IL-10* expression (J, K, L); and *TGF-β1* expression (M, N, O), by senescent and non-senescent pulp cells. Heatmap indicating the variation in *IDO, TNF-α, IL-6, IL-10*, and *TGF-β1* expression (P). Experimental conditions: Untreated cultures (UT), LPS-stimulated cultures (+LPS), and LPS plus IFN-γ-stimulated cultures (+LPS+ IFN-γ). *p <0.0245, **p <0.0013 and ****p <0.0001, by one-way ANOVA test.

CAPÍTULO 3.

Figure 1. Diagram of the induced senescence protocol and its confirmation by β-galactosidase staining assay, along with peptide treatment. (A) Peptide treatment prior senescence induction. (B) Peptide treatment after senescence induction.

Figure 2. Screening of a library of 15 peptides at different concentrations using the β-galactosidase staining assay, with peptide treatment prior to senescence induction by doxorubicin. Bars represent the following groups: Untreated, 50 μM, 12.5 μM, 5 μM, and 500 nM, respectively. Percentage above each bar indicates the proportion of β-galactosidase-positive cells in each condition, in relation to the untreated group. Peptides: (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, (F) C6, (G) C7, (H) C8, (I) C9, (J) C10, (K) C11, (L) C12, (M) C13, (N) C14, (O) C15. *p < 0.05, **p < 0.005, ***p < 0.0005, by one-way ANOVA test.

Figure 3. Screening of a library of 15 peptides at different concentrations using the β-galactosidase staining assay, with peptide treatment after senescence induction by doxorubicin. Bars represent the following groups: Untreated, 50 μM, 12.5 μM, 5 μM, and 500 nM, respectively. Percentage above each bar indicates the proportion of β-galactosidase-positive cells in each condition, in relation to the untreated group. Peptides: (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, (F) C6, (G) C7, (H) C8, (I) C9, (J) C10, (K) C11, (L) C12, (M) C13, (N) C14, (O) C15. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, by one-way ANOVA test.

Figure 4. Cell viability of pulp cells treated with a library of 15 peptides at different concentrations, before (1) and after (2) senescence induction. Cell viability of the peptide-treated groups under both conditions was as follows: (A1 and A2) C1, (B1 and B2) C2, (C1 and C2) C3, (D1 and D2) C4, (E1 and E2) C5, (F1 and F2) C6, (G1 and G2) C7, (H1 and H2) C8, (I1 and I2) C9, (J1 and J2) C10, (K1 and K2) C11, (L1 and L2) C12, (M1 and M2) C13, (N1 and N2) C14, (O1 and O2) and C15. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ by one-way ANOVA test.

Figure 5. Nitric oxide production of pulp cells treated with a library of 15 peptides at different concentrations, before (1) and after (2) senescence induction. Nitrite levels in the peptide-treated groups under both conditions were as follows: (A1 and A2) C1, (B1 and B2) C2, (C1 and C2) C3, (D1 and D2) C4, (E1 and E2) C5, (F1 and F2) C6, (G1 and G2) C7, (H1 and H2) C8, (I1 and I2) C9, (J1 and J2) C10, (K1 and K2) C11, (L1 and L2) C12, (M1 and M2) C13, (N1 and N2) C14, (O1 and O2) and C15. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ by one-way ANOVA test.

Supplementary Figure 1. Spectrum obtained by MALDI-ToF of peptides C1, C2, C3, and C4.

Supplementary Figure 2. Spectrum obtained by MALDI-ToF of peptides C5, C6, C7, and C8.

Supplementary Figure 3. Spectrum obtained by MALDI-ToF of peptides C9, C10, C11, and C12.

Supplementary Figure 4. Spectrum obtained by MALDI-ToF of peptides C13, C14 and C15.

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Anexo 2. Trabalhos publicados durante o período de mestrado.

Anexo 3. Trabalhos em fase de submissão, elaborados durante o período de mestrado.

LISTA DE ABREVIATURAS E SIGLAS

SA-β-gal – *senescence-associated beta-galactosidase* (enzima lisossomal associada à senescência β-galactosidase)

SASP – *senescence-associated secretory phenotype* (fenótipo secretor associado a senescência)

DNA – *deoxyribonucleic acid* (ácido desoxirribonucleico)

CO_2 – *carbon dioxide* (dióxido de carbono)

X-gal – *5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside* (5-bromo-4-chloro-3-indolil-β-D-galactopiranosídeo)

DMEM – *dulbecco's Modified Eagle Medium* (meio eagle modificado por dulbecco)

DMSO – *dimethyl sulfoxide* (dimetilsulfóxido)

EDTA – *ethylenediamine tetraacetic acid* (ácido etilenodiamino tetraacético)

GAPDH – *glyceraldehyde 3-phosphate dehydrogenase* (gliceraldeído-3-fosfato desidrogenase)

IFN-γ – *interferon-gama* (nterferon-gama)

LPS – *lipopolysaccharide* (lipopolissacarídeo)

MALDI – *matrix-assisted laser desorption/ionization* (ionização e dessorção a laser assistida por matriz)

MEV – microscopia eletrônica de varredura

SEM – *scanning electron microscopy*

MTT – *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio brometo)

NO – *nitric oxide* (óxido nitrico)

ROS – *reactive oxygen species* (espécies reativas de oxigênio)

PBS – *phosphate-buffered saline* (solução salina tamponada com fosfato)

qPCR – *quantitative polymerase chain reaction* (reação em cadeia da polimerase quantitativa)

RNA – *ribonucleic acid* (ácido ribonucleico)

SFB – soro fetal bovino

FSB - *fetal bovine serum*

IDO - *indoleamine 2, 3-dioxygenase* (indolamina-pirrole 2,3-dioxigenase)

TNF-α - *tumor necrosis fator- α* (fator de necrose tumoral-α)

TGF-β1 - *transforming growth factor beta 1* (fator de transformação do crescimento beta 1)

IL – *interleukin* (interleucina)

TCLE - termo de consentimento livre e esclarecido

IA – inteligência artificial

AI - *artificial intelligence*

ML – *machine learning* (aprendizado de máquina)

RL - *reinforcement learning* (aprendizado por reforço)

GRAMPA - *giant repository of amp activities* (repositório que contém informações sobre peptídeos antimicrobianos)

CNN - *convolutional neural networks* (rede neural convolucional)

MLP - *multilayer perceptron* (perceptron multicamada)

LSTM-LM - *long short-term memory-language models* (modelo de linguagem baseado em LSTM)

PPO - *proximal policy optimization* (otimização de política proximal)

ESM-2 - *evolutionary scalability modeling* (modelagem em escala evolutiva)

VOSViewer - *visualization of similarities viewer* (visualizador de similaridades)

CAAE - certificado de apresentação para apreciação ética

SUMÁRIO

PREFÁCIO

A dissertação de mestrado aqui apresentada dá continuidade aos esforços do nosso grupo de pesquisa na investigação do processo de senescência celular, com enfoque no contexto pulpar. A ideia para este trabalho surgiu em meados de 2020, a partir do estudo que gerou a publicação do artigo *"Senescence on Dental Pulp Cells: Effects on Morphology, Migration, Proliferation, and Immune Response"*, desenvolvido pela aluna Jade, minha antiga colega de grupo. Esse estudo inicial exerceu uma influência significativa na construção da pesquisa atual, pois, ao buscarmos caracterizar a senescência celular e observarmos seu impacto nos tratamentos endodônticos conservadores, tornou-se evidente a necessidade de aprofundarmos nossos conhecimentos sobre o tema. Além disso, identificamos a importância de explorar esse processo em contextos mais complexos e investigar estratégias para atenuar seus efeitos.

Durante minha iniciação científica, tive a oportunidade de participar do Programa de Iniciação Científica (PIBIC) como bolsista CNPq. Sob a orientação da Profa. Taia Rezende, ainda na Universidade Católica de Brasília, iniciei meu trabalho no Biodonto, onde não apenas desenvolvi habilidades em pesquisa, mas também aprendi a valorizar o trabalho em equipe e a importância das parcerias. Essa experiência me mostrou o valor do compartilhamento de conhecimento no ambiente acadêmico. Foi nesse contexto que tive o primeiro contato com o tema da senescência celular e comecei a idealizar meu projeto de mestrado. Em 2023, conclui minha graduação em Odontologia pela UCB, no mesmo ano em que ingressei no Programa de Pós-Graduação em Ciências da Saúde, como bolsista CAPES.

Assim, o conteúdo desta dissertação foi estruturado em consonância com o meu aprendizado sobre o tema e as demandas de pesquisa na área. O Capítulo 1 marca meus primeiros passos na exploração da senescência celular, apresentando uma revisão abrangente da literatura. Nele, são abordados os mecanismos moleculares da senescência, seu impacto na cavidade bucal e no tecido pulpar, além da influência de produtos odontológicos na indução desse processo e estratégias para sua modulação. À medida que o trabalho avançava e meu conhecimento sobre o tema se aprofundava, tornou-se evidente a necessidade de um levantamento bibliométrico.

Assim, optamos por desenvolver uma revisão mais ampla, incorporando elementos de revisão de escopo e análise bibliométrica.

Já com uma bagagem teórica adquirida nos primeiros meses de mestrado, após um período intenso de estudos e a aprovação ética, prossegui para a segunda fase da minha pesquisa. O Capítulo 2 apresenta os resultados dos meus primeiros experimentos como aluno de mestrado, nos quais pude aplicar, na prática, todo o conhecimento teórico e observacional adquirido durante minha iniciação científica. Nesse período, realizei meus primeiros isolamentos e culturas celulares a partir da extração do tecido pulpar de terceiros molares hígidos, utilizando a técnica de explante. Os experimentos seguiram uma sequência lógica de caracterização e grau de dificuldade, abrangendo ensaios de indução e confirmação da senescência, indução de ambientes inflamatórios complexos *in vitro*, microscopia eletrônica de varredura, ensaios de *scratch*, proliferação, viabilidade celular e expressão gênica. Fui motivado por um grande interesse nos efeitos do processo de senescência e seu impacto no sucesso de uma gama de tratamentos atualmente explorados na endodontia, especialmente aqueles investigados pelo nosso grupo, como tratamentos pulpares conservadores e terapia endodôntica regenerativa.

Por fim, o Capítulo 3 surgiu da necessidade de explorar novas terapias para mitigar a senescência na polpa dentária, um campo ainda inexplorado na odontologia. Dentre os três capítulos, esse talvez tenha sido o mais desafiador, tanto pela escassez de referências técnicas quanto pela complexidade das análises e experimentos, que envolveram a triagem de múltiplos grupos de peptídeos. A ideia para esse estudo nasceu de uma série de conversas entre mim, minha orientadora, Profa. Taia Rezende, e o Prof. Octávio Franco, um grande colaborador do nosso grupo, que já trabalhou no desenvolvimento do peptídeo senoterapêutico OS-01 (ou Peptídeo 14), atualmente disponível no mercado de cosméticos. No entanto, a falta de sequências conhecidas de peptídeos senoterapêuticos nos levou a buscar alternativas para o desenho e triagem dessas moléculas. Foi nesse contexto que surgiu a colaboração com Abel Ley, aluno de mestrado orientado pelo Prof. Octávio Franco e coorientado pelo Prof. Fabiano Fernandes que também trabalhava no desenvolvimento e estudo de novos peptídeos. Apesar de nossas pesquisas terem finalidades distintas, nosso grupo já havia explorado a versatilidade dos peptídeos antimicrobianos para diferentes aplicações, incluindo atividade imunomodulatória. Assim, fui incumbido de testar os

mesmos 15 peptídeos desenvolvidos no estudo do Abel, avaliando seu potencial senoterapêutico. Para isso, utilizei técnicas de coloração histoquímica, ensaios de citotoxicidade e dosagem de NO, permitindo a investigação do potencial dessas moléculas na modulação da senescência celular.

Esta dissertação só se tornou realidade graças ao apoio e a colaboração de inúmeras pessoas e instituições que, ao longo desta jornada acadêmica, contribuíram de diferentes formas para seu desenvolvimento. A cada um que esteve presente, oferecendo conhecimento, incentivo e suporte, expresso minha mais profunda gratidão. A pesquisa científica é um caminho desafiador, repleto de incertezas, aprendizados e conquistas, e essa trajetória não teria sido possível sem a orientação de professores dedicados, o trabalho conjunto com colegas e colaboradores, e o suporte de instituições que acreditam na importância da ciência. Cada contribuição, seja por meio de discussões, trocas de ideias, auxílio técnico ou mesmo palavras de encorajamento, teve um papel fundamental na construção deste trabalho. Espero que os conhecimentos aqui apresentados sirvam como base para futuras investigações, incentivando novas descobertas e aprimorando estratégias terapêuticas. Mais do que um registro acadêmico, desejo que este estudo contribua para o avanço da área e, sobretudo, para a melhoria da qualidade de vida dos pacientes que poderão se beneficiar das inovações e dos avanços científicos.

INTRODUÇÃO

A senescência e o envelhecimento estão intimamente relacionados, mas não são sinônimos. O envelhecimento é um processo natural e gradual, previsível no organismo humano, decorrente de mecanismos pró-envelhecimento, como danos ao DNA, peroxidação lipídica e dobramento incorreto de proteínas. Esses processos levam à morte ou senescência celular. A senescência celular, por sua vez, caracteriza-se pela interrupção irreversível do ciclo celular. Na maioria dos casos, as células senescentes interrompem a duplicação na fase G1, embora certos oncogenes possam induzir a senescência na fase G2 (Kudlova et al., 2022). Apesar de não se dividirem, essas células permanecem vivas e metabolicamente ativas, contribuindo para o envelhecimento e para patologias associadas à idade avançada. Em contraste, células não senescentes são comumente denominadas "jovens" ou "em estado proliferativo" (Campisi et al., 2007).

Desde os estudos de Hayflick e Moorhead, em 1961, que demonstraram o limite de aproximadamente 60 divisões para células humanas primárias, a senescência tornou-se um tema central na pesquisa biomédica (Hayflick et al., 1961). Mecanismos moleculares relacionados à senescência, como vias associadas e potenciais alvos terapêuticos, têm sido amplamente investigados (Campisi et al., 2007; Kuilman et al., 2010). Recentemente, o foco tem se ampliado para a eliminação seletiva de células senescentes, visando rejuvenescimento tecidual e alívio de doenças relacionadas à idade (Yosef et al., 2016; Cai et al., 2020). Assim, a definição de senescência evoluiu, abrangendo atualmente condições celulares desencadeadas por fatores intrínsecos e extrínsecos, como ativação oncogênica, estresse oxidativo, disfunção mitocondrial, radiação e quimioterápicos (Kuilman et al., 2010).

A senescência celular pode ser classificada em replicativa, causada pelo encurtamento dos telômeros, ou induzida por estresse (Shay et al., 2000). Telômeros disfuncionais desencadeiam a senescência por meio da via p53, aumentando a expressão de p16 e ativando a via p16-retinoblastoma. Além da interrupção do ciclo celular, as células senescentes apresentam alterações características, como mudanças estruturais, aumento do volume celular, vacúolos ricos em citoplasma, maior massa mitocondrial, alterações nucleares e aumento de conteúdo lisossomal. A

atividade da enzima β-galactosidase associada à senescência é frequentemente utilizada como marcador típico desse estado (Campisi et al., 2007).

Embora inicialmente a senescência tenha evoluído como um mecanismo protetor para limitar a proliferação de células danificadas, sabe-se que contribui para o envelhecimento, para o desenvolvimento de diversas doenças relacionadas à idade e para o declínio funcional geral (Weiskopf et al., 2009). No sistema imunológico, essas alterações levam à imunossenescência, caracterizada por declínio progressivo das funções imunológicas e aumento da susceptibilidade a doenças. A imunossenescência afeta tanto o sistema imunológico inato quanto o adaptativo, sendo este último mais significativamente alterado. Esse estado impacta subpopulações celulares, padrões de secreção de citocinas e tolerância imunológica, além de comprometer a eliminação de células senescentes (Krabbe et al., 2004).

Além disso, o fenótipo secretor associado a senescência (SASP), que se caracteriza pela produção e secreção de uma grande quantidade de compostos pró-inflamatórios, representa uma das principais manifestações da senescência celular. Essa secreção excessiva de mediadores inflamatórios compromete várias funções do sistema imunológico, incluindo a capacidade de eliminar eficazmente as células senescentes, o que pode resultar em uma acumulação dessas células no organismo. Ademais, o SASP exerce um efeito parácrino, induzindo a senescência nas células adjacentes, o que amplifica o processo de senescência celular e contribui para a perpetuação da inflamação local (Rodrigues et al., 2021).

Outro conceito relacionado ao envelhecimento é o "*inflammaging*", que descreve uma inflamação crônica, de baixo grau e estéril, associada ao envelhecimento. Esse estado inflamatório é um fator de risco significativo para morbidade e mortalidade em pessoas idosas, devido ao papel central da inflamação na patogênese de diversas doenças. Além disso, essa condição pode comprometer a resposta imune, reduzindo a capacidade do organismo de combater infecções e prejudicando a regeneração tecidual, impactando negativamente a saúde geral dos indivíduos (Franceschi et al., 2000).

Dessa forma, a cavidade oral representa um dos muitos locais onde a senescência atua, levando ao aumento da inflamação local e contribuindo para inúmeras patologias orais. A polpa dentária é um tecido altamente vascularizado e

inervado, que confere vitalidade aos dentes e desempenha diversas funções na manutenção da saúde dental. No entanto, sabe-se que a senescência pulpar é acompanhada por várias alterações morfológicas e funcionais nesse tecido, resultando na perda ou diminuição de suas capacidades (Farias et al., 2024).

No tecido pulpar humano, células desempenham funções imunomodulatórias que também são afetadas pela senescência (Franceschi et al., 2014). Com o envelhecimento, há uma redução da capacidade regenerativa, sensorial, imunológica e de nutrição da polpa dentária, além de alterações estruturais, como diminuição de tamanho e volume da polpa, calcificações arteriais, redução do número de células, diminuição da circulação sanguínea e da sensibilidade pulpar. Fatores estressantes, como cárie crônica, fraturas dentárias, traumas oclusais e bruxismo, também podem induzir senescência celular na polpa (Farias et al., 2023).

Adicionalmente, o *inflammaging* na polpa dentária resulta em secreção aumentada de citocinas pró-inflamatórias, como o TNF-α, que promove a mineralização do tecido pulpar (Pezelj-Ribaric et al., 2002). Estudos relatam encurtamento dos telômeros, redução da atividade da fosfatase alcalina, menor potencial de proliferação e diferenciação celular, menor resistência à apoptose e aumento da autofagia nas células pulpares senescentes (Shiba et al., 2003; Iezzi et al., 2019; Zhai et al., 2017; Li et al., 2012). Essas alterações podem comprometer tratamentos clínicos conservadores, como o capeamento pulpar, devido ao impacto direto na funcionalidade das células do tecido pulpar (Farias et al., 2024).

Além disso, a exposição prolongada a produtos odontológicos potencialmente nocivos ao tecido pulpar pode comprometer a integridade celular, acelerando o processo de senescência. Nesse contexto, é fundamental que a pesquisa odontológica explore com mais profundidade os efeitos desses materiais nos tecidos dentários, visando compreender melhor suas implicações e desenvolver abordagens terapêuticas seguras. Sabe-se que certos adesivos dentários têm o potencial de inibir a proliferação celular e desencadear respostas inflamatórias, enquanto monômeros residuais de resinas compostas e fotoiniciadores podem gerar espécies reativas de oxigênio, contribuindo para o estresse celular e favorecendo possíveis mutações genéticas (Kazak et al. 2024; Sürmelioğlu et al. 2020).

No mais, clareadores dentários à base de peróxido de hidrogênio, também vem se mostrando potencialmente prejudiciais a homeostase do tecido pulpar, contribuindo para a senescência. Esses produtos representam um risco significativo, pois possuem a capacidade de induzir estresse oxidativo, danificar o DNA das células pulpares e desencadear uma resposta inflamatória exacerbada na polpa dentária. Esse conjunto de efeitos adversos pode comprometer a saúde do tecido pulpar, acelerando o processo de senescência celular e prejudicando a capacidade de regeneração do tecido (Torres et al. 2021).

Portanto, é crucial a realização de pesquisas focadas na prevenção e/ou no tratamento da senescência celular. Diversos estudos têm destacado o potencial de estratégias como a utilização de antioxidantes, como as vitaminas C e E, que ajudam a neutralizar as espécies reativas de oxigênio (ROS) (Ryan et al. 2010). Outra abordagem promissora envolve o controle dos telômeros, com a telomerase desempenhando um papel fundamental na manutenção do comprimento telomérico e no retardamento da senescência. No entanto, é importante ressaltar que sua ativação desregulada pode aumentar o risco de desenvolvimento de câncer (Rossiello et al. 2022; Xu et al. 2016).

Além disso, a modulação das vias de sinalização celular, como as vias p53/p21 e p16INK4a/Rb, tem sido investigada, e compostos como resveratrol e rapamicina mostraram potencial para reduzir a senescência precoce (Prieur et al. 2011; Bian et al. 2020). O uso de senolíticos e senomórficos, substâncias que eliminam ou modificam as células senescentes, também está em estudo, com evidências de que senolíticos como quercetina podem melhorar a função tecidual. Por fim, o uso de peptídeos como GHK-Cu pode estimular a regeneração celular e modular a expressão de genes envolvidos na reparação dos tecidos (Pickart et al. 2018).

Dentre as estratégias focadas em mitigar a senescência, o peptídeo FOXO4-DRI se destaca, sendo um antagonista projetado para interromper a interação entre FOXO4 e p53, promovendo a exclusão nuclear de p53 e sua realocação para as mitocôndrias, onde induz a apoptose nas células senescentes (Baar et al., 2017). Vários estudos demonstraram o efeito terapêutico de FOXO4-DRI na eliminação de células senescentes (Huang et al., 2021; Meng et al., 2021; Li et al., 2024). Outrossim, mesmo diante do comprometimento do tecido, o FOXO4-DRI é capaz de restaurar

efetivamente a homeostase, promovendo a recuperação funcional e estrutural do tecido afetado.

Não obstante, o dasatinibe e a quercetina demonstraram eficácia na redução dos níveis de senescência celular em pacientes com doença renal diabética. A administração desses senolíticos contribui para uma diminuição da carga de células senescentes tanto no tecido adiposo quanto na pele, além de promover melhorias na função dos podócitos renais, reduzir a resistência à insulina e diminuir a presença de proteína na urina. Esses efeitos sugerem o potencial terapêutico desses compostos para tratar complicações associadas à senescência celular em condições metabólicas (Hickson et al., 2019).

O peptídeo OS-01 (ou peptídeo 14) demonstrou alta eficácia na redução da carga de senescência em modelos tridimensionais de pele humana e em amostras de pele *ex vivo*, promovendo marcadores de saúde e diminuindo significativamente a idade biológica da pele. Em um estudo piloto com 22 mulheres entre 47 e 65 anos, uma formulação contendo o peptídeo OS-01 apresentou resultados clínicos promissores, melhorando a função da barreira cutânea, a textura e a radiância da pele, além de atenuar a profundidade das rugas. Esses resultados destacam o OS-01 como um tratamento senoterapêutico promissor para combater os sinais visíveis do envelhecimento da pele (Zonari et al., 2024).

No mais, o peptídeo UBX0101, um inibidor da interação entre p53 e MDM2, foi identificado como um potencial indutor de apoptose em células senescentes. No estudo de Lane et al. (2021), o UBX0101 foi administrado por via intra-articular em pacientes com osteoartrite dolorosa no joelho. Embora o tratamento não tenha mostrado uma redução estatisticamente significativa na dor ou na melhora funcional, os dados apresentados indicam que o peptídeo pode influenciar a diminuição das células senescentes na articulação, o que pode ajudar na modulação da inflamação e na melhoria dos sintomas em doenças degenerativas articulares (Lane et al., 2021).

Os peptídeos desempenham um papel essencial em diversas funções biológicas dos organismos vivos, regulando inúmeros processos fisiológicos e mentais, como os hormônios. No cérebro humano, já foram identificados mais de 60 peptídeos diferentes, cada um com funções específicas. No mais, essas moléculas são formadas pela ligação de dois ou mais aminoácidos por meio de ligações

peptídicas. Graças à sua versatilidade estrutural e funcional, os peptídeos têm sido amplamente estudados no campo científico e em aplicações terapêuticas (Wimley et al., 2010).

O potencial biotecnológico dos peptídeos vem sendo explorado para modular respostas imunológicas, combater infecções, tratar patologias e promover a regeneração de tecidos (Wang et al., 2016). Uma das principais vantagens dos peptídeos como agentes terapêuticos é sua alta seletividade e especificidade de ação. Isso ocorre devido à capacidade dos peptídeos de se ligar a receptores específicos ou interagir de maneira precisa com proteínas-alvo. Essa característica permite um direcionamento mais eficaz das terapias, reduzindo significativamente o risco de efeitos adversos, tornando os peptídeos promissores em muitas áreas da saúde (Wimley et al., 2010; Wang et al., 2016).

O design racional de peptídeos tem sido impulsionado pelo avanço de tecnologias inovadoras que possibilitam a criação de moléculas bioativas com elevada especificidade e eficácia (Kaynak, 2021). Anteriormente, a descoberta e o aprimoramento de peptídeos dependiam de abordagens experimentais demoradas, baseadas em processos de tentativa e erro, o que tornava a otimização dessas biomoléculas um desafio prolongado e de alto custo (Mata et al., 2023). No entanto, a incorporação de ferramentas computacionais sofisticadas revolucionou esse campo, permitindo uma abordagem mais precisa e eficiente (Hashemi et al., 2024).

Nesse contexto, a inteligência artificial (IA) e os algoritmos de aprendizado de máquina (ML) têm transformado a descoberta de medicamentos ao possibilitar a identificação de padrões complexos em grandes volumes de dados. Além disso, redes neurais generativas viabilizam a criação de novas estruturas moleculares com propriedades específicas, acelerando o desenvolvimento de terapias inovadoras para diversas doenças. Por fim, algoritmos de aprendizado por reforço (RL) aprimoram processos na descoberta de medicamentos, incluindo triagem virtual e ancoragem molecular, tornando-os mais eficientes e precisos (Lavecchia, 2019).

Essas estratégias estão em constante avanço e podem desempenhar um papel fundamental na modulação da senescência celular. No entanto, a aplicação da IA no desenvolvimento de senoterapêuticos enfrenta desafios significativos, sendo um dos principais a escassez de conjuntos de dados de alta qualidade sobre sequências de

peptídeos, essenciais para o treinamento eficaz de modelos preditivos (Li et al., 2022). Para superar essas limitações, a pesquisa biomédica tem explorado moléculas consideradas promíscuas, ou seja, capazes de interagir com múltiplos alvos celulares e modular diversas vias biológicas (Silva et al., 2011).

Atualmente, uma variedade de peptídeos com ação antimicrobiana tem sido amplamente descrita, com suas estruturas e mecanismos de ação detalhadamente compreendidos (Kumar et al., 2018). No entanto, como discutido anteriormente, o foco tem se deslocado para o desenvolvimento de peptídeos que, além de possuírem suas propriedades originais, no caso antimicrobianas, também desempenham outras funções biológicas. Esses peptídeos multifuncionais estão sendo investigados por sua capacidade de não apenas combater microrganismos patogênicos, mas também modular processos celulares essenciais (Amorim et al., 2021; Lima et al., 2017).

Devido às suas propriedades físico-químicas, como carga positiva e anfipaticidade, os peptídeos antimicrobianos interagem de forma eficaz com as membranas celulares, o que facilita a penetração em componentes intracelulares e potencializa sua ação (Kumar et al., 2018). Essa capacidade de interagir com as membranas e vias celulares pode, além disso, influenciar diversos processos celulares que estão envolvidos na senescência celular, como a regulação do estresse oxidativo e a modulação da inflamação, ambos fatores chave nesse processo (Hilchie et al., 2013; Van Harten et al., 2018).

Assim, a pesquisa tem se concentrado cada vez mais em peptídeos capazes de modular diversos processos biológicos. No entanto, apesar dos esforços contínuos para mitigar a senescência celular, ainda não existem estudos que descrevam o efeito de senoterapêuticos no complexo dentino-pulpar. Esta dissertação de mestrado, por meio de seus três capítulos, tem como principal objetivo explorar a literatura científica em busca dos principais mecanismos da senescência celular e seu impacto no complexo dentino-pulpar. Além disso, são abordados os produtos odontológicos usados na prática clínica diária que induzem esse processo, bem como as estratégias para modular a senescência. No mais, buscamos validar o impacto desse processo na polpa dentária por meio de diversos experimentos *in vitro*, com o intuito de avaliar sua influência na resposta imune e de reparo do tecido pulpar, sob diferentes condições inflamatórias. O trabalho também inclui o desenho e a triagem de novas moléculas com potencial senoterapêutico.

OBJETIVOS

Objetivo Geral

Avaliar a viabilidade, as alterações morfológicas, a capacidade proliferativa e migratória, e a resposta imunológica de células humanas senescentes derivadas da polpa dentária, comparando-as com as células não senescentes sob diferentes condições inflamatórias. Além disso, investigar a identificação de peptídeos com potencial ação senoterapêutica.

Objetivos Específicos

(1) Disponibilizar uma visão geral dos estudos sobre senescência na odontologia, com ênfase no contexto dentino-pulpar, abordando aspectos como o número de publicações, as citações, as áreas de concentração, os tipos de estudo e as palavras-chave mais relevantes.

(2) Explorar e relatar os produtos odontológicos que induzem a senescência no tecido pulpar, bem como os mecanismos propostos na literatura para mitigar esse estado.

(3) Induzir *in vitro* a senescência em células da polpa dentária humana por meio do tratamento com doxorrubicina, visando replicar condições senescentes.

(4) Avaliar a indução da senescência nas células pulpares utilizando o ensaio de β-galactosidase como marcador de senescência celular.

(5) Induzir *in vitro* um modelo imunoinflamatório mediado por LPS ou LPS com IFN-γ, para mimetizar as possíveis consequências da senescência celular na polpa dentária em situações de saúde e de inflamação/infecção.

(6) Avaliar alterações relacionadas a morfologia de células pulpares senescentes e não senescentes por meio de microscopia eletrônica de varredura (MEV).

(7) Avaliar a atividade migratória, proliferativa e a viabilidade de culturas de células pulpares senescentes em comparação com culturas de células pulpares não senescentes.

(8) Avaliar a resposta imune de células senescentes e não senescentes, por meio da expressão de mediadores inflamatórios, incluindo a *Indolamina-pirrole 2,3-*

dioxigenase (*IDO*), o *fator de necrose tumoral* (*TNF-α*), o *fator de transformação do crescimento beta* (*TGF-β1*) e as *interleucinas (IL)-6* e *IL-10*, utilizando qPCR.

(9) Avaliar o potencial senoterapêutico de peptídeos desenhados *in silico*, testando sua eficácia em culturas de células pulpares antes e após indução da senescência, utilizando o ensaio de β-galactosidase para monitorar a modulação da senescência.

(10) Avaliar a toxicidade dos peptídeos selecionados em células pulpares antes e após a indução da senescência.

(11) Investigar a atividade dos peptídeos selecionados na produção de óxido nítrico (NO) em culturas de células pulpares antes e após a indução da senescência.

MATERIAIS E MÉTODOS

Delineamento Experimental

O presente estudo foi estruturado em três capítulos. O primeiro capítulo consistiu em uma revisão bibliométrica sobre a senescência no complexo dentino-pulpar, abordando produtos capazes de induzir ou mitigar esse processo no tecido pulpar. O segundo capítulo envolveu uma avaliação quantitativa e qualitativa *in vitro* dos efeitos da senescência celular em células pulpares humanas, com análise da viabilidade celular, morfologia, capacidade proliferativa e migratória, além da resposta imunoinflamatória. Para isso, foram utilizadas condições inflamatórias induzidas por LPS ou LPS e IFN-γ, mimetizando as possíveis consequências da senescência celular na polpa dentária em cenários de saúde, inflamação e infecção. Por fim, o terceiro capítulo incluiu a triagem de uma biblioteca de 15 peptídeos projetados por inteligência artificial, utilizando ensaios histoquímicos para detecção de beta-galactosidase, avaliação da viabilidade celular pelo ensaio de MTT e quantificação da produção de óxido nítrico pelo método de Griess (Figura 1).

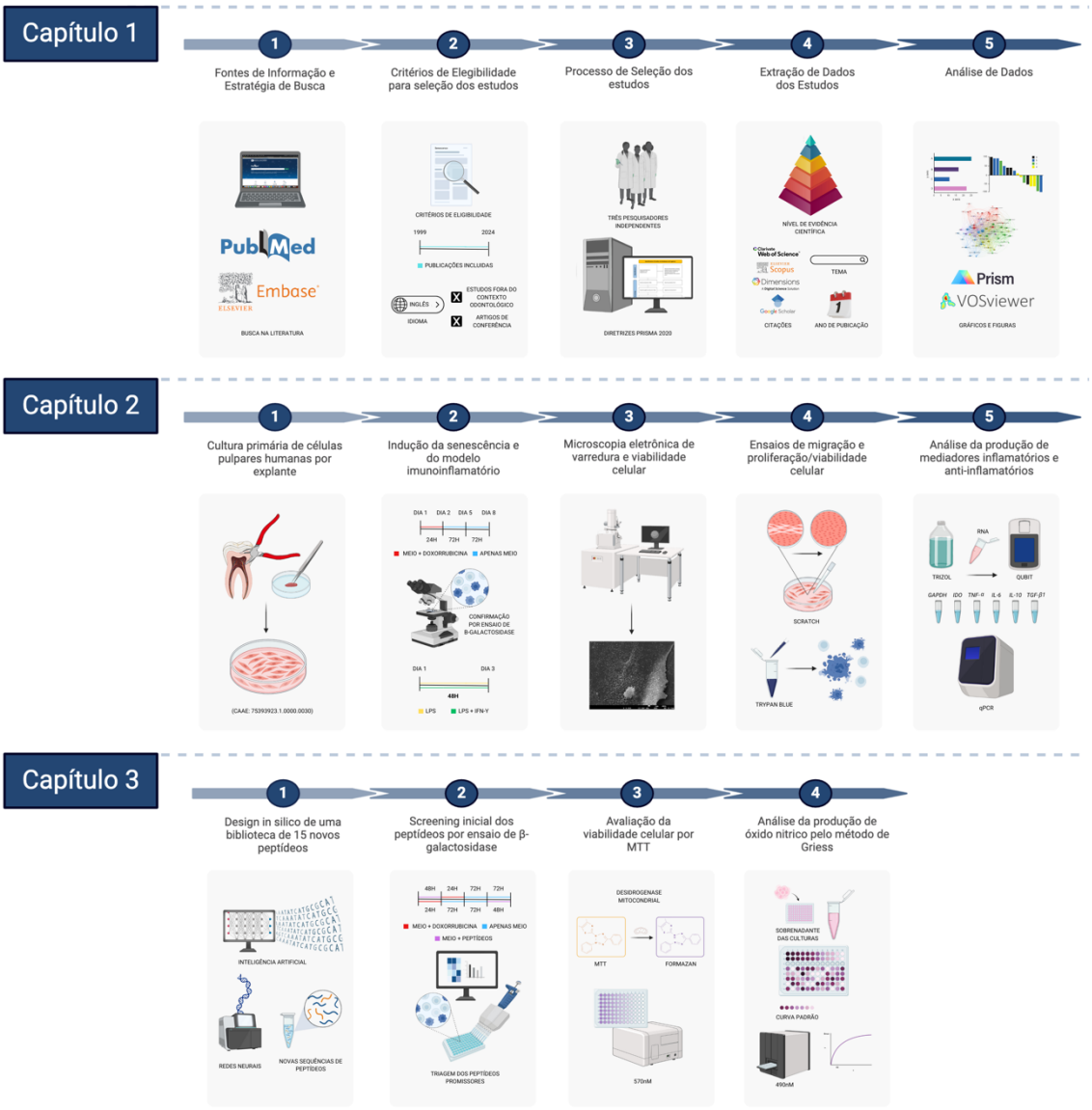

Figura 1. Fluxograma representativo dos três capítulos da pesquisa.

Capítulo 1

Fontes de Informação e Estratégia de Busca

Uma busca eletrônica foi realizada em 17 de outubro de 2024, nas bases de dados PubMed e Embase (https://pubmed.ncbi.nlm.nih.gov/ e https://www.embase.com/search/quick). A seleção dos artigos seguiu a estratégia de busca: ("*Cellular Senescence*" OR "*Senescence*" OR "*Aging*" OR ("*Senescence*" AND "*Dentistry*") OR ("*Senescence*" AND "*Oral Health*") OR ("*Senescence*" AND

"*Periodontal Diseases*") OR ("*Senescence*" AND "*Pulp Cells*") OR ("*Senescence*" AND "*Endodontic Treatment*") OR ("*Aging*" AND "*Oral Health*") OR ("*Aging*" AND "*Periodontal Diseases*"). Para refinar a busca, foram considerados os *Medical Subject Headings* (MeSH), bem como sinônimos e termos relevantes relacionados à senescência e odontologia mencionados em artigos relacionados. Nenhum filtro foi aplicado, permitindo uma busca abrangente.

Critérios de Elegibilidade

Esta análise bibliométrica incluiu estudos que investigaram, descreveram ou mencionaram a senescência em contextos relacionados à odontologia. Foram selecionados artigos em inglês, publicados entre 1999 e 2024. Publicações que não abordaram a senescência no contexto odontológico, bem como artigos de conferências, foram excluídos.

Processo de Seleção

Os artigos foram selecionados por três pesquisadores independentes (J.C.S., M.E.F.M. e R.F.R.) após a análise do título, resumo e/ou texto completo, quando necessário. Os desacordos foram resolvidos por consenso com um quarto pesquisador (T.M.B.R.). Após a busca nas bases de dados, 1.871 registros foram inicialmente identificados. Após a remoção de duplicatas e registros marcados como inelegíveis por ferramentas de automação, 1.456 estudos permaneceram para a fase de triagem. Durante a análise dos títulos e resumos, 1.069 estudos foram excluídos por não atenderem aos critérios de inclusão. Assim, 387 estudos foram selecionados para avaliação de elegibilidade do texto completo. Destes, 279 foram posteriormente excluídos, cada um por razões específicas. Por fim, 108 estudos publicados entre 1999 e 2024 foram incluídos nesta revisão (Figura 2).

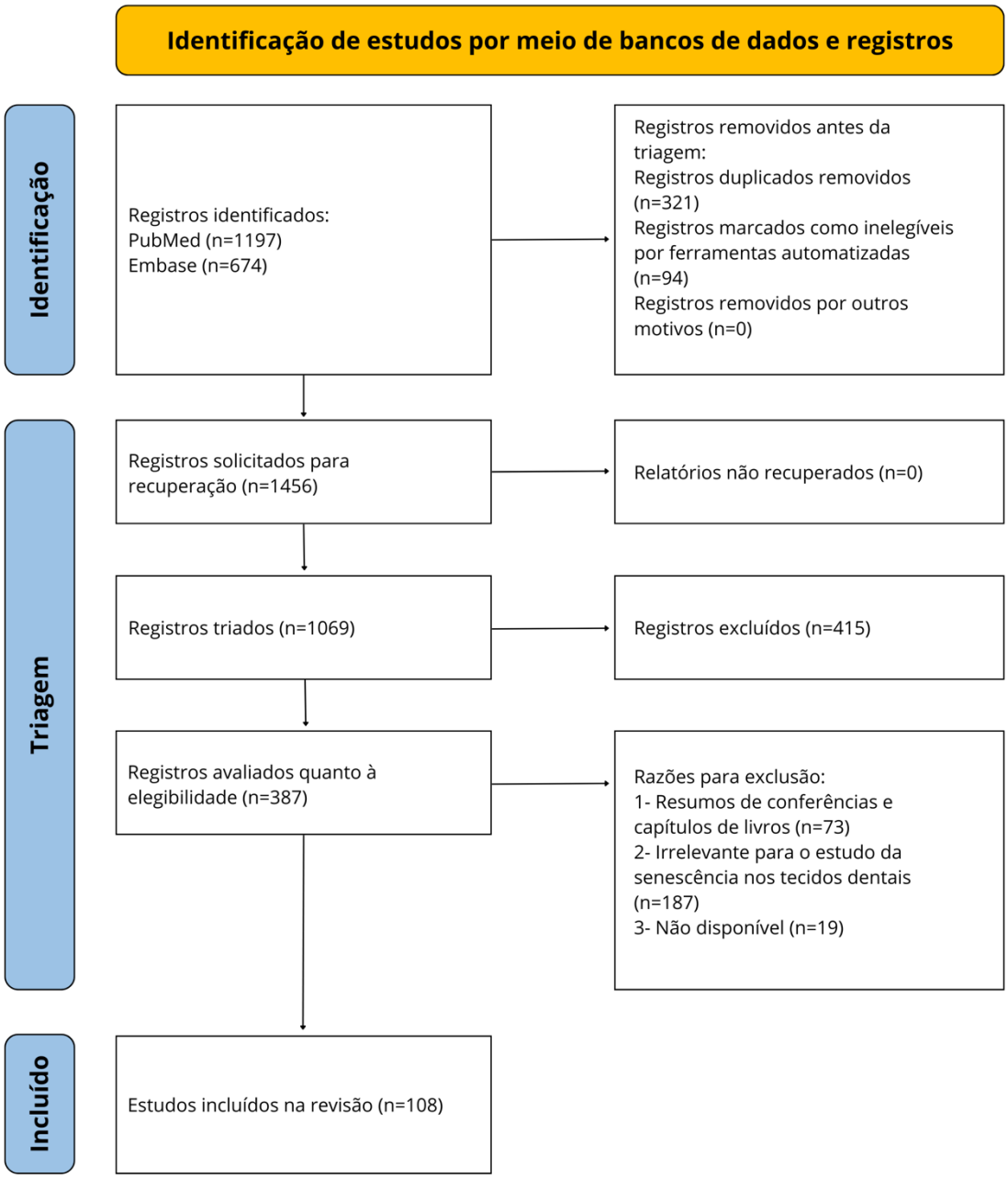

Figura 2. Diagrama do fluxo de busca na literatura e critérios de seleção adaptados do PRISMA 2020.

Extração de Dados dos Estudos Selecionados

Os seguintes dados bibliométricos foram extraídos de cada artigo: número de citações, ano de publicação, tipo de estudo, tópico e palavras-chave. Os tipos de estudo foram classificados em *In Vitro*, *In Vivo*, *In vitro/In vivo*, Clínico, Revisões de Literatura, Revisões Sistemáticas, Revisões Bibliométricas, Coorte, Caso-Controle e Transversal. Com base nos tópicos abordados nos estudos, estes foram agrupados de acordo com os temas mais prevalentes, a saber: Polpa Dentária, Ligamento

Periodontal, Glândulas Salivares, Câncer Oral, Queratinócitos Orais, Gengiva, Cavidade Oral, Mucosa, Folículo Dentário, Papila Apical e Periodonto, sendo posteriormente categorizados em subcategorias: Formação Estrutural, Diagnóstico/Patogênese, Tratamento, Prevenção e Prognóstico. Os dados extraídos foram transferidos para o Microsoft Excel 2024 (Microsoft, Redmond, WA) para categorização.

Análise de Dados

A representação gráfica das redes bibliométricas foi realizada utilizando o *software Visualization of Similarities Viewer* (VOSViewer) (versão 1.6.20, Países Baixos), o qual permitiu a identificação das palavras-chave mais frequentes. Por outro lado, o *software Graph Pad Prism* 5 foi utilizado para a preparação de outros gráficos. Na análise da rede, os termos associados aos maiores pontos focais e fontes apresentaram maior frequência de ocorrência. Em contraste, os termos ligados a pontos focais ou fontes menores mostraram ocorrência reduzida. As linhas conectando os termos indicam colaborações entre eles.

Capítulo 2

Cultura Primária de Células Pulpares Humanas

Os tecidos pulpares foram obtidos a partir de terceiros molares hígidos extraídos de doadores com idades entre 18 e 30 anos, após aprovação do comitê de ética da Universidade de Brasília (CAAE: 75393923.1.0000.0030). Em uma capela de fluxo laminar, os dentes foram lavados com solução salina tamponada com fosfato (PBS) e fraturados utilizando alicates ortodônticos (Quinelato, Rio Claro, São Paulo, Brasil). A polpa dental coronal e radicular foi cuidadosamente removida utilizando limas manuais tipo K (Dentsply Sirona, Charlotte, Carolina do Norte, EUA). As polpas dentárias foram lavadas com PBS contendo 100 $U.mL^{-1}$ de penicilina (Invitrogen, Grand Island, Nova York, EUA), 100 $\mu g.mL^{-1}$ de estreptomicina (Invitrogen), e 1% de anfotericina B (Invitrogen). Em seguida, o tecido pulpar foi dilacerado com uma lâmina de bisturi e fixado em placas de cultura de células de 6 poços (Kasvi, São José dos Pinhais, Paraná, Brasil). O meio *Dulbecco's Modified Eagle's Medium* (DMEM) (Sigma

Aldrich, San Luis, Missouri, EUA), suplementado com 20% de soro fetal bovino (SFB) (GIBCO, Grand Island, Nova York, EUA), 100 $\mu g.mL^{-1}$ de penicilina (Invitrogen), 100 $\mu g.mL^{-1}$ de estreptomicina (Invitrogen) e 2 $\mu g.mL^{-1}$ de glutamina (GIBCO) foi adicionado a cada poço. As placas foram mantidas em uma atmosfera umidificada contendo 5% de CO_2 à temperatura de 37 °C. As células foram subcultivadas à medida que atingiam a confluência para obter células suficientes para cada experimento (Naz et al., 2019).

Indução da Senescência Celular e Condições Imuno-inflamatórias

A senescência celular foi induzida *in vitro* por doxorrubicina (Doxorubicin hydrochloride, Sigma Aldrich). Para isso, as células pulpares foram incubadas com 500 µM de doxorrubicina (Sigma Aldrich) em meio DMEM (Sigma Aldrich) suplementado (conforme descrito anteriormente) por 24 horas. Em seguida, o meio foi trocado para o meio DMEM (Sigma Aldrich) suplementado com 10% de soro fetal bovino (SFB) (GIBCO), com trocas subsequentes de meio a cada 72 horas, durante 7 dias. O grupo não senescente foi representado por células incubadas com meio DMEM suplementado (Sigma Aldrich). Após o protocolo de senescência celular, as células foram lavadas duas vezes por 30 segundos com PBS. Imediatamente após, as células foram fixadas com uma solução fixadora [2% de formaldeído (Dinâmica, Indaiatuba, São Paulo, Brasil) e 0,5% de glutaraldeído (Dinâmica)], por 5 minutos à temperatura ambiente, e então lavadas novamente com solução PBS. Em seguida, 1 mL da solução de coloração X-Gal [solução de ácido cítrico 40 mM (Dinâmica) e fosfato de sódio (Sigma Aldrich), hexacianoferrato de potássio 5 mM (Sigma Aldrich), cloreto de sódio 150 mM (Sigma Aldrich), cloreto de magnésio 2 mM (Sigma Aldrich), 1 $mg.mL^{-1}$ de X-Gal (Thermo Fisher Scientific, Waltham, Massachusetts, EUA)] foi adicionado e a placa foi incubada a 37 °C, protegida da luz, durante a noite. Após 16 horas, as células foram lavadas duas vezes por 30 segundos com PBS e uma vez com metanol (Dinâmica). Em seguida, as células foram observadas e fotografadas na região central de cada poço, sempre com a mesma ampliação (4X), utilizando um microscópio invertido com contraste de fase Axio Observer D1 (Zeiss, Oberkochen, Alemanha). Células coradas e não coradas foram contadas utilizando o software Image J (NIH, Bethesda, Maryland, EUA) (Yaghoobi et al., 2020). Para simular diferentes condições imuno-inflamatórias *in vitro*, foram adicionados LPS (1 $\mu g.mL^{-1}$)

(lipopolissacarídeos de Escherichia coli, Sigma) e IFN-γ (1 μg.mL^{-1}) (Interferon-gama, Sigma) como estímulos, seja individualmente ou combinados, por 24 horas (Hong et al., 2021).

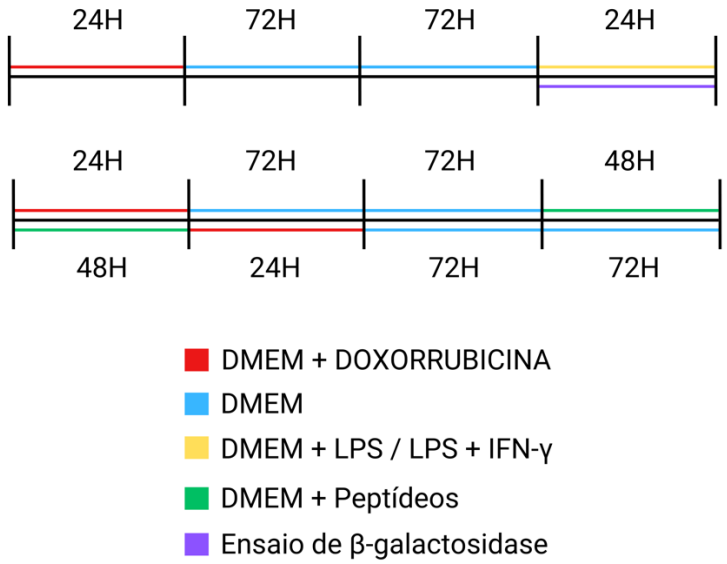

Figura 3. Diagrama esquemático do protocolo de indução da senescência e sua confirmação por ensaio de coloração com β-galactosidase, juntamente com o modelo *in vitro* do sistema imuno-inflamatório e tempos de tratamento com os peptídeos.

Microscopia Eletrônica de Varredura (MEV)

As alterações morfológicas foram analisadas por Microscopia Eletrônica de Varredura (MEV). Células pulpares não senescentes e senescentes (2x10^5 células por poço), estimuladas com diferentes condições imuno-inflamatórias, foram semeadas em placas de cultura de 6 poços (Kasvi) com meio DMEM (Sigma Aldrich) suplementado (como descrito anteriormente). Lâminas de vidro redondas de 13 mm × 13 mm (Fisher Scientific, Suwanee, GA) foram colocadas no fundo das placas antes da cultura celular. As lâminas de vidro foram fixadas em solução de Karnovsky 0,1M (2% de glutaraldeído e paraformaldeído) por 24 horas, seguidas de duas lavagens com solução de cacodilato de sódio 0,1M. As células foram então fixadas em tetróxido de ósmio 1% por 30 minutos e lavadas duas vezes com água destilada. A desidratação foi realizada em soluções de acetona a 50%, 70%, 90% e 100%. Após a secagem das lâminas de vidro, estas foram metalizadas e analisadas utilizando um microscópio eletrônico de varredura (JSM 7001F, Jeol). Imagens foram capturadas com

ampliações de 1500x, 3000x e 5000x. Para a análise, foram selecionadas áreas contendo no mínimo 10 células, assegurando maior robustez estatística. A análise quantitativa foi realizada quanto ao tamanho das células e ao número de extensões celulares utilizando o software ImageJ (NIH, Bethesda, MD) (Fig. 2A) (Santos et al., 2019).

Migração Celular (*Scratch*)

A migração celular foi realizada pelo método de arranhadura ou *Scrath*. Células pulpares não senescentes e senescentes ($2,5x10^5$ células por poço), estimuladas com diferentes condições imuno-inflamatórias, foram semeadas em placas de cultura de 6 poços (Kasvi) com meio DMEM (Sigma Aldrich) suplementado (como descrito anteriormente). As células foram mantidas até que uma camada confluente fosse formada. Uma raspagem da porção central da placa de cultura foi realizada utilizando uma ponta de micropipeta de 1000 µL com uma abertura grande. Em seguida, o meio de cultura foi trocado para DMEM (Sigma Aldrich) sem SFB (GIBCO). As culturas foram incubadas e monitoradas por até 48 horas. Fotografias foram tiradas utilizando microscopia aos 0, 24 e 48 horas para análise posterior (Martinotti et al., 2019). As imagens foram processadas, e as células na área da ferida foram contadas utilizando o *software Image J* (NIH).

Viabilidade Celular e Proliferação

Células pulpares senescentes e não senescentes ($2x10^4$ células) estimuladas com diferentes condições imuno-inflamatórias foram cultivadas em meio DMEM (Sigma Aldrich) sem SFB (GIBCO), em placas de 96 poços (Kasvi). Após o período de incubação, as células foram ressuspendidas, e uma solução de coloração com azul de tripano a 0,4% (Sigma Aldrich) foi adicionada por 1 minuto. As células vivas e mortas foram imediatamente contadas após 24 e 48 horas (Croley et al., 2016).

Produção de Mediadores Inflamatórios e Anti-inflamatórios

A expressão de *IDO, TNF-α, IL-6, IL-10* e *TGF-β1* por células pulpares senescentes e não senescentes foi avaliada utilizando qPCR em tempo real. Os grupos de células senescentes e não senescentes foram avaliados sob diferentes condições imuno-inflamatórias, bem como em condições basais (culturas não estimuladas). Após o cultivo celular, o RNA foi extraído pelo método TRIzol™ (ThermoFisher Scientific, Califórnia, EUA) (5). Em seguida, a quantificação do RNA foi determinada pelo Qubit® (6). A análise da expressão gênica foi realizada por qPCR em tempo real utilizando o sistema StepOnePlus™ Real-Time PCR System (ThermoFisher) para verificar a expressão de genes indicativos de processos inflamatórios e anti-inflamatórios. Para essa análise, a expressão gênica foi avaliada. GAPDH foi usado como gene de referência. A quantificação foi realizada conforme as recomendações do fabricante. Cada reação teve um volume final de 10 µL, composto por: 5 µL de SYBR Green; 0,5 µL de primers, com 0,25 µL de primer para cada gene (Tabela 1); e 2,5 µL de água livre de nucleases (ThermoFisher Scientific - SYBR™ Green PCR Master Mix KIT). Os níveis de expressão relativos no grupo experimental foram calculados utilizando o método Δ(ΔCT)(ΔCTControl − ΔCTExperiment) (Livak et al., 2001).

Gene	Forward	Reverse
GAPDH	TCAACGACCACTTTGTCAAGCTCAGCT	GGTGGTCCAGGGGTCTTAC
IDO	GGGAAGCTTATGACGCCTGT	CTGGCTTGCAGGAATCAGGA
TNF-α	CACAGTGAAGTGCTGGCAAC	GATCAAAGCTGTAGGCCCCA
IL-6	TCAATATTAGAGTCTCAACCCCCA	TTCTCTTTCGTTCCCGGTGG
IL-10	GGTGGTCCAGGGGTCTTAC	ACTCTGCTGAAGGCATCTCG
TGF-β1	GCTGTATTTAAGGACACCGTGC	TGACACAGAGATCCGCAGTC

Tabela 1. Sequência dos primers para cada gene utilizado no ensaio de qPCR. *Gliceraldeído 3-fosfato desidrogenase* (*GAPDH*); *Indolamina-pirrole 2,3-dioxigenase* (*IDO*); *Fator de necrose tumoral alfa* (*TNF-α*); *Interleucina-6* (*IL-6*); *Interleucina-10* (IL-10); *Fator de crescimento transformante beta 1* (*TGF-β1*).

Capítulo 3

Design *in silico* dos peptídeos

A sequência dos peptídeos escolhidos foi projetada por inteligência artificial por um grupo colaborador e seguiu os seguintes passos: foram utilizadas bases de dados de sequências de peptídeos disponíveis para treinar e testar modelos preditivos. Um conjunto de dados positivo, com 684 sequências, foi extraído do GRAMPA (*Giant Repository of AMP Activities*) e filtrado usando o CD-hit para eliminar redundâncias. Adicionalmente, um conjunto de dados negativo foi obtido do banco de dados *UniProt*, excluindo resíduos não padrão, também filtrado pelo CD-hit e reduzido aleatoriamente para 684 sequências para formar um conjunto final de dados. Ambos os conjuntos de dados foram unificados, totalizando 1.368 sequências para o treinamento do modelo. Em seguida, uma função de pontuação foi desenvolvida para prever a atividade antimicrobiana, e uma rede neural convolucional (CNN) foi implementada como codificador, com uma camada de perceptron multicamada (MLP) para classificação binária. O banco de dados criado foi dividido aleatoriamente em 80% para treinamento e 20% para avaliação, sendo utilizado para treinamento e avaliação do modelo. Após isso, o espaço conformacional da atividade antimicrobiana foi definido, limitando o comprimento da sequência a 16 aminoácidos, restringindo o ácido aspártico a zero e a lisina a pelo menos três, com o objetivo de obter peptídeos catiônicos com maior atividade. O processo é iterativo, ajustando as restrições para gerar variantes eficazes. O agente utilizado para gerar novas sequências foi um modelo LSTM-LM (Long Short-Term Memory - Modelos de Linguagem), treinado na base de dados *SwissProt* e ajustado para gerar sequências com atividade antimicrobiana usando o banco de dados positivo. O método de gradiente PPO (*Proximal Policy Optimization*) foi empregado, e a estabilidade das sequências geradas foi avaliada com o modelo ESM-2. A recompensa atribuída às sequências, com base na classificação e estabilidade, varia de -10 a 10. O modelo AMP-BERT, baseado na API *Hugging Face Transformers* e ProtBERT-BFD, foi utilizado para validar a classificação das sequências geradas. Após o ajuste fino com o banco de dados criado anteriormente, o modelo foi treinado e avaliado (80% e 20%, respectivamente) para prever a atividade antimicrobiana. Sequências com probabilidades ≥ 0,5 foram classificadas como antimicrobianas, e aquelas com probabilidades mais baixas, como não

antimicrobianas. Sequências com recompensas > 9,5 no modelo MRL e previsões ≥ 0,8 pelo AMP-BERT foram selecionadas. O ToxinPred 3.0 e o HemoPred descartaram sequências tóxicas ou hemolíticas. Apenas sequências com carga líquida ≥ +2 foram retidas (Fernandes et al., 2023). Com essa metodologia, 15 peptídeos foram selecionados (sequências não listadas por proteção intelectual) (Tabela 2).

Peptídeo	Fórmula Molecular	Espectro de massa
C1	$C_{65}H_{119}N_{19}O_{17}$	1438,71
C2	$C_{63}H_{114}N_{18}O_{14}$	1347,61
C3	$C_{69}H_{127}N_{21}O_{14}$	1474,82
C4	$C_{67}H_{128}N_{22}O_{14}$	1466,04
C5	$C_{65}H_{117}N_{21}O_{16}$	1449,17
C6	$C_{65}H_{120}N_{20}O_{17}S$	1486,41
C7	$C_{61}H_{109}N_{19}O_{15}$	1349.80
C8	$C_{63}H_{117}N_{19}O_{19}$	1445,83
C9	$C_{63}H_{116}N_{22}O_{16}S$	1469,49
C10	$C_{66}H_{125}N_{19}O_{16}S$	1472,71
C11	$C_{66}H_{125}N_{19}O_{17}S$	1488,74
C12	$C_{63}H_{119}N_{19}O_{17}S_2$	1476,84
C13	$C_{66}H_{125}N_{21}O_{15}$	1453,22
C14	$C_{64}H_{119}N_{21}O_{14}$	1407,37
C15	$C_{59}H_{109}N_{19}O_{14}$	1309,47

Tabela 2. Uma biblioteca de 15 peptídeos com potencial antimicrobiano, incluindo o nome do peptídeo, fórmula molecular e espectro de massa.

Síntese de peptídeos e determinação da massa molecular por espectrometria de massas MALDI-ToF

Os 15 peptídeos selecionados foram adquiridos da empresa SYNBIOTech (New York, USA) com uma pureza de >95%. Os peptídeos purificados foram analisados qualitativamente utilizando espectrômetro de massa MALDI-ToF UltraFlex III (Bruker Daltonics). Para as análises, os peptídeos liofilizados foram dissolvidos em água ultrapura, misturados em uma solução saturada de uma matriz constituída por

ácido α- ciano-4-hidroxicinâmico (1:3), depositados em uma placa do tipo *Anchorchip* e deixados cristalizar à temperatura ambiente. A calibração foi realizada utilizando Peptide calibration standard II (Bruker Daltonics) como padrões de massa molecular e adquiridos em modo de operação refletido positivo (RP_700-3000 Da).

Screening da biblioteca de peptídeos

Como vários peptídeos possuem mais de uma atividade, sendo frequentemente chamados de peptídeos promíscuos, o potencial senoterapêutico desses peptídeos foi avaliado. Para isso, foi realizada uma triagem inicial da biblioteca de 15 peptídeos obtidos, analisando a presença de moléculas senoterapêuticas por meio da coloração de beta-galactosidase associada à senescência (SA-BGal) em células pulpares humanas antes e após a indução de senescência com doxorrubicina a 500 µM (Doxorubicin hydrochloride, Sigma Aldrich) (Figura 3). As células pulpares foram semeadas em placas de 96 poços (Kasvi) a uma densidade de $2x10^4$ células/poço. A biblioteca de peptídeos foi adicionada em concentrações finais de 50 µM, 12,5 µM, 5 µM e 500 nM em DMEM (Sigma Aldrich) sem SFB. Após 30 minutos, 10% de SFB (GIBCO) foi adicionado à cultura. Em seguida, as células foram incubadas por 48 horas a 37 °C e 5% de CO_2. Células senescentes foram usadas como controle positivo, e células não senescentes foram consideradas controle negativo, ambas sem tratamento com peptídeos. Nos grupos experimentais, após o tratamento com peptídeos, as células foram lavadas duas vezes por 30 segundos com PBS. Imediatamente depois, as células foram fixadas com 2% de formaldeído (Dinâmica) e 0,5% de glutaraldeído (Dinâmica), por 5 minutos à temperatura ambiente. Subsequentemente, 1 mL da solução de coloração X-Gal [solução de ácido cítrico 40 mM (Dinâmica) e fosfato de sódio (Sigma Aldrich), hexacianoferrato de potássio 5 mM (Sigma Aldrich), cloreto de sódio 150 mM (Sigma Aldrich), cloreto de magnésio 2 mM (Sigma Aldrich), 1 $mg.mL^{-1}$ X-Gal (Thermo Fisher Scientific)] foi adicionado, e a placa foi incubada a 37 °C, protegida da luz, durante a noite. Após 16 horas, as células foram lavadas duas vezes por 30 segundos com PBS e uma vez com metanol (Dinâmica). Em seguida, as células foram observadas e fotografadas na região central de cada poço, sempre com a mesma ampliação (10X), utilizando um microscópio invertido com contraste de fase Axio Observer D1 (Zeiss, Oberkochen, Alemanha). O nível relativo

de senescência foi quantificado usando o software CellProfiler (Cimini Lab, Cambridge, Massachusetts, EUA) (Zonari et al., 2023).

Viabilidade celular por MTT

Para avaliar a viabilidade celular após 48 horas de incubação com os peptídeos, foi utilizado o ensaio colorimétrico MTT (Sigma-Aldrich). Este método baseia-se na avaliação da atividade da enzima desidrogenase mitocondrial. Após o período de incubação, o sobrenadante foi removido e 100 µL de meio DMEM (Sigma-Aldrich) foi adicionado por poço. Em seguida, 10 µL de MTT (5 $mg \cdot mL^{-1}$) foram adicionados a cada poço. As placas foram incubadas por 4 horas em 5% CO_2 a 37 °C e 95% de umidade. Após esse período, a reação foi bloqueada com a adição de 60 µL de dimetilsulfóxido (DMSO; Sigma-Aldrich) por poço e homogenizada para garantir a solubilização completa do conteúdo celular. A absorbância foi então medida a 570 nm utilizando um leitor Elisa (Bio-Tek PowerWave HT, EUA). A viabilidade celular nos grupos experimentais foi comparada com o grupo controle positivo, representado pela cultura celular no meio (considerado 100% de viabilidade). O controle negativo foi representado pela cultura celular em solução de lise (10 mM Tris pH 7,4, 1 mM EDTA e 0,1% Triton X-100) (Mosmann et al., 1983).

Produção de óxido nítrico

Após 48 horas de incubação celular, o sobrenadante da cultura obtido de ambos os grupos, antes e após a indução de senescência com doxorrubicina (Sigma Aldrich), foi coletado e avaliado quanto à presença de NO. A produção de nitrito nos sobrenadantes da cultura foi avaliada utilizando a reação de Griess, e assumiu-se que refletia os níveis de NO. Em resumo, 50 µL de sobrenadantes de cultura foram colocados em placas de 96 poços, seguidos pela adição de 50 µL de uma mistura contendo 1% de sulfanilamida (Sigma-Aldrich) em 2,5% de ácido fosfórico (Vetec, Brasil) e 1% de N-(1-naftil)etilenodiamina (Sigma-Aldrich) em 2,5% de ácido fosfórico (Vetec), na proporção de 1:1. Após 10 minutos de incubação à temperatura ambiente, a leitura foi realizada no leitor ELISA (Bio-Tek PowerWave HT) a 490 nm. A quantidade

de nitrito foi calculada utilizando uma curva padrão de nitrito de sódio (200 μM–1,5625 μM) (Green et al., 1982).

Análise Estatística

Todos os experimentos foram realizados em triplicatas técnicas e biológicas. Os dados foram descritos como médias e desvios padrão. A normalidade dos dados foi verificada pelo teste de Shapiro-Wilk, e, de acordo com essa análise, a estatística apropriada foi aplicada. A indução de senescência foi analisada estatisticamente pelo teste *t* de *Student*, enquanto os ensaios de migração celular, proliferação, viabilidade, imunomodulatórios e a triagem da biblioteca de peptídeos foram analisados por *one-way* ANOVA. Todas as análises foram realizadas utilizando o *software Graph Pad Prism* 10 e consideradas com um nível de significância de 95%.

CAPÍTULO 1 (Intended submission: Biogerontology; IF = 4.4)

Dental products and dentin-pulp complex senescence: an in-depth analysis

ABSTRACT

Cellular senescence is a complex biological process characterized by irreversible cell cycle arrest. Initially viewed as a protective mechanism against damaged cells and tumorigenesis, it is now understood to contribute to aging and age-related diseases. Although senescence protects against cellular dysfunction, it can also trigger processes such as inflammation and degeneration. In dental tissues, especially in the pulp, senescence leads to morphological and functional changes, affecting tissue vitality and the success of conservative endodontic treatments. These changes are driven by factors such as bruxism, caries, and trauma, which promote oxidative stress and inflammation, accelerating cellular aging. Dental products, such as adhesives, restorative materials, and whitening agents, can cause cellular damage, contributing to senescence. This highlights the need for safer clinical applications. Following the PRISMA 2020 guidelines, an electronic search was conducted in PubMed and Embase databases (October 17, 2024) to identify studies on cellular senescence in dental contexts (1999–2024). After screening 1,871 records, 108 studies were included. Bibliometric data were extracted and analyzed. The review highlighted the increase in publications on cellular senescence in dental tissues, especially after 2021. Most studies focus on pathogenesis, with gaps in therapeutic approaches. Additionally, the need to investigate the effects of dental materials on senescence and new drug proposals with senotherapeutic potential was emphasized. Therefore, this review explored the mechanisms of cellular senescence in the dentin-pulp complex and identified possible strategies to minimize its effects, which may lead to greater preservation and regeneration of the tissue, as well as an improvement in the prognosis of conservative endodontic treatments.

Keywords: Senescence; Pulp cells; Dental Materials; Senotherapeutics.

INTRODUCTION

Cellular senescence is a complex biological process characterized by an irreversible disruption of the cell cycle and has become a prominent topic in contemporary biomedical research. Initially described as an essential mechanism to prevent the proliferation of damaged cells and suppress tumorigenesis, senescence is now widely recognized as playing a dual role. While it acts as a protective barrier against tumor processes and cellular dysfunctions, it also significantly contributes to aging and progression of various age-related diseases (Rodrigues et al., 2021). Growing evidence highlights the intricate interaction between cellular senescence and systemic homeostasis, associating this phenomenon with diverse pathophysiological conditions, such as inflammatory and degenerative diseases (Farias et al., 2023).

Among the tissues impacted by senescence, dental pulp stands out due to its unique structural and functional characteristics. This tissue, highly vascularized and innervated, plays crucial roles in oral health, including sensory, immune, and reparative functions. However, with aging and exposure to chronic stressors such as caries, trauma, and restorative materials, the pulp undergoes morphological and functional changes. These changes are driven by the accumulation of senescent cells, compromising not only the vitality of the tissue but also the effectiveness of conservative and regenerative endodontic therapies (Galler et al., 2021).

Recent molecular biology and dentistry research has advanced the understanding of the underlying mechanisms of pulp senescence. Among the characteristic markers, notable ones include telomere shortening, increased expression of senescence-associated genes such as p21 and p16, and the manifestation of a senescence-associated secretory phenotype (SASP) (Rodrigues et al., 2021; Huang et al., 2022). The latter contributes to heightened local inflammation and accelerated tissue degeneration, impairing pulp functionality (Farias et al., 2023). Despite these advances, there are still significant gaps in understanding this process, especially regarding strategies to prevent or minimize the effects of senescence on dental tissues.

Furthermore, using dental products such as restorative materials, desensitizing agents, and whitening agents may pose risks to dental tissues, particularly the dentin-

pulp complex. Compounds released by dentin adhesives, photoinitiators, and composite resins can induce oxidative stress, inflammation, and cellular damage, promoting cellular senescence (Koruyucu et al., 2024; D'Alpino et al., 2017). Whitening products containing hydrogen peroxide also contribute to these effects (Liu et al., 2025). These findings emphasize the need for further research to understand the impact of these materials better and guide safer clinical applications (Kazak et al., 2024; Alvarez et al., 2019).

In this context, recent research has focused on methods to prevent or treat senescence, such as reducing oxidative stress through antioxidants like vitamins C and E (Ryan et al., 2010) and targeting telomeres with controlled activation of telomerase to delay senescence (Rossiello et al., 2022). Additionally, modulation of cellular signaling pathways, such as the p53/p21 and p16INK4a/Rb pathways, with compounds like resveratrol and rapamycin, may help reduce premature senescence (Bian et al., 2020). Advances in the development of senolytic compounds, which promote the selective elimination of senescent cells, and senomorphic compounds, which modulate their phenotypic characteristics without inducing apoptosis, have demonstrated therapeutic potential (Zhang et al., 2022). Peptides like GHK-Cu are being explored for their regenerative potential (Pickart et al., 2018). These strategies present promising perspectives for promoting tissue health and healthy aging (Zonari et al., 2023).

This study aims to deepen the understanding of the mechanisms involved in cellular senescence in the dentin-pulp complex, investigating its biological and clinical implications. The goal of analyzing the factors that trigger and modulate this process is to better understand its consequences for dental health, particularly regarding the functionality and integrity of dental tissues. Moreover, by exploring potential therapeutic approaches, the study seeks to identify strategies that may minimize the senescence effects, promoting the preservation and regeneration of the dentin-pulp complex and, consequently, improving the outcomes of conservative endodontic treatments.

MATERIAL AND METHODS

Information Sources and Search Strategy

An electronic search was conducted on October 17, 2024, in the PubMed and Embase databases (https://pubmed.ncbi.nlm.nih.gov/ and https://www.embase.com/search/quick). The article selection followed the search strategy: ("Cellular Senescence" OR "Senescence" OR "Aging" OR ("Senescence" AND "Dentistry") OR ("Senescence" AND "Oral Health") OR ("Senescence" AND "Periodontal Diseases") OR ("Senescence" AND "Pulp Cells") OR ("Senescence" AND "Endodontic Treatment") OR ("Aging" AND "Oral Health") OR ("Aging" AND "Periodontal Diseases"). To narrow the search, Medical Subject Headings (MeSH), as well as synonyms and relevant terms related to senescence and dentistry, mentioned in related articles, were considered. No filters were applied, allowing for a comprehensive search.

Eligibility Criteria

This bibliometric analysis included studies that investigated, described, or mentioned senescence in contexts related to dentistry. Articles in English, published between 1999 and 2024, were selected. Publications that did not address senescence in the dental context, as well as conference papers, were excluded.

Selection Process

Articles were selected by three independent researchers (J.C.S., M.E.F.M., and R.F.R.) after reviewing the title, abstract, and/or full text when necessary. Disagreements were resolved by consensus with a fourth researcher (T.M.B.R.). After database search, 1,871 records were initially identified. After the removal of duplicates and records marked as ineligible by automation tools, 1,456 studies remained for the screening phase. Upon examining the titles and abstracts, 1,069 studies were excluded for not meeting the inclusion criteria. Thus, 387 studies were selected for a full-text eligibility assessment. Of these, 279 reports were subsequently excluded, each

for specific reasons. Finally, 108 studies published between 1999 and 2024 were included in this review (Figure 1).

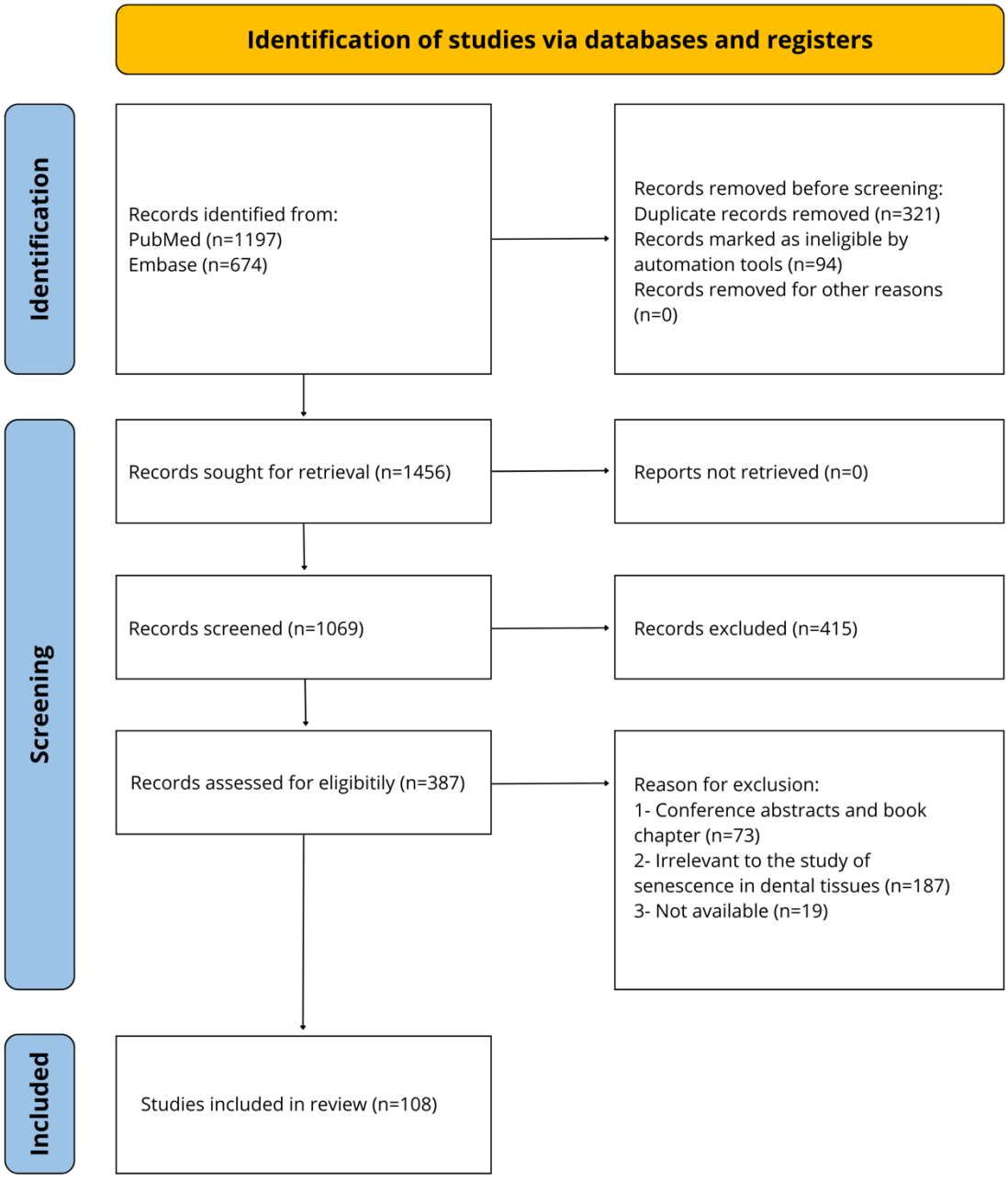

Figure 1. Flow diagram of literature search and selection criteria adapted from PRISMA 2020.

Data Extraction from Selected Studies

The following bibliometric data were extracted from each article: number of citations, year of publication, study type, topic, and keywords. Study types were classified into *In Vitro*, *In Vivo*, *In vitro/In vivo*, Clinical, Literature Reviews, Systematic

Reviews, Bibliometric Reviews, Cohort, Case-Control, and Cross-Sectional. Based on the topics addressed in the studies, they were grouped according to the most prevalent themes, namely: Dental Pulp, Periodontal Ligament, Salivary Glands, Oral Cancer, Oral Keratinocytes, Gingiva, Oral Cavity, Mucosa, Dental Follicle, Apical Papilla, and Periodontium, and further categorized into subcategories: Structural Formation, Diagnosis/Pathogenesis, Treatment, Prevention, and Prognosis. Extracted data were transferred to Microsoft Excel 2024 (Microsoft, Redmond, WA) for categorization.

Data Analysis

Graphical representation of the bibliometric networks was performed using the Visualization of Similarities Viewer (VOSViewer) software (version 1.6.20, Netherlands), which allowed for the identification of the most frequent keywords. On the other hand, Graph Pad Prism 5 software was used for the preparation of other graphs. In the network analysis, terms associated with the largest focal points and sources exhibited higher frequency of occurrence. In contrast, terms linked to smaller focal points or sources showed reduced occurrence. The lines connecting the terms indicate collaborations between them.

MECHANISMS OF CELLULAR SENESCENCE

The mechanisms of cellular senescence have been discussed among researchers worldwide, but the literature still clearly lacks information regarding this important cellular process (Rodrigues et al. 2021). One of the most common definitions of senescence is linked to the physiological aging of individuals. However, despite being a complementary process, aging is a natural and gradual mechanism of the organism. At the same time, senescence is a complex cellular event, defined by irreversible cell cycle interruption, leading to numerous changes in cell biology (Kudlova et al. 2022).

Although cellular senescence is believed to have evolved to control the proliferation of old and/or damaged cells, this process has contributed to aging, age-related diseases, and general deterioration of cellular functions (Farias et al., 2023).

Furthermore, intrinsic and extrinsic factors can lead to a cellular senescence stage by a mechanism that is already known. Thus, senescence can be divided into replicative senescence and premature senescence. The former is associated with the cellular replication threshold (Hayflick limit) and is caused by telomere shortening during cell division (Reed et al., 2023).

Furthermore, this process can also be induced by extrinsic stressful factors, such as cellular DNA damage, oxidative stress, and epigenetic alterations, with this pathway known as premature senescence (Yousefzadeh et al. 2021). It should also be noted that the accumulation of damage to a cell's genetic material can trigger a DNA repair response; if the damage is extensive and cannot be repaired, mitosis is prevented, and cells are induced to undergo apoptosis or cellular senescence (which is a state resistant to apoptosis). In all conditions, senescence cells act by attracting and activating immune cells, which ultimately leads to their elimination (Rodrigues et al. 2021).

Yet, during the physiological aging of the organism, there is a significant increase in senescent cells, and the elimination of these cells by the immune system does not occur efficiently. Moreover, the accumulation of senescent cells can propagate senescence by recruiting healthy cells by adopting a senescence-associated secretory phenotype (SASP), which paracrinely induces these cells to become senescent (Zonari et al. 2023). Thus, this cellular mechanism is accompanied by an imbalance in cellular homeostasis, leading to genetic, metabolic, and signaling changes.

Additionally, some typical senescent cell markers include cellular morphology alterations (size and shape of cells), telomere shortening, as well as changes in the cellular nucleus, which may involve an increase in nucleus size and irregularities in chromatin (Huang et al., 2022). Furthermore, there is an increased expression of p21, p16, and p53 genes, high levels of reactive oxygen species, and an increase in β-galactosidase enzyme activity, highly associated with senescence (Farias et al. 2024). Senescent cells also exhibit metabolic alterations, including dysregulation of the mTOR pathway and impaired autophagy, leading to the accumulation of damaged organelles and proteins (Cayo et al., 2022). Additionally, the activation of the DNA damage response (DDR) and the persistent presence of the senescence-associated secretory phenotype (SASP) contribute to the reinforcement of the senescent state and the

propagation of pro-inflammatory signals to surrounding tissues (Wu et al., 2023; Ohtani et al., 2022). All these factors contribute to functional modifications in cells, including increased inflammation and a decrease in the ability of these cells to regenerate and repair tissue (Kumari et al. 2021).

Therefore, it is crucial to understand the molecular mechanisms underlying the process of cellular senescence for the development of approaches to prevent and regulate this state (Roger et al. 2021). It significantly contributes to a high susceptibility to infectious diseases and age-related conditions, as well as impacting vascularization, which is relevant in the pulp tissue. In summary, it is necessary to thoroughly explore how this state relates to inflammation and immunosenescence, as it is known that senescence affects both innate and adaptive immune responses, as well as causing alterations in the response of numerous tissues (Liu et al. 2023).

With this, the oral cavity represents one of the numerous sites where senescence acts, leading to an increase in local inflammation and contributing to numerous oral pathologies. Pulp is a highly vascularized and innervated tissue that provides vitality to the teeth and has various functions for maintaining dental health. However, it is known that pulp senescence is accompanied by several morphological and functional changes in this tissue, losing or decreasing its capacities (Figure 2). Ultimately, this can lead to dysfunctions that result in the failure of conservative endodontic treatments and especially of regenerative endodontic therapy (Farias et al. 2024).

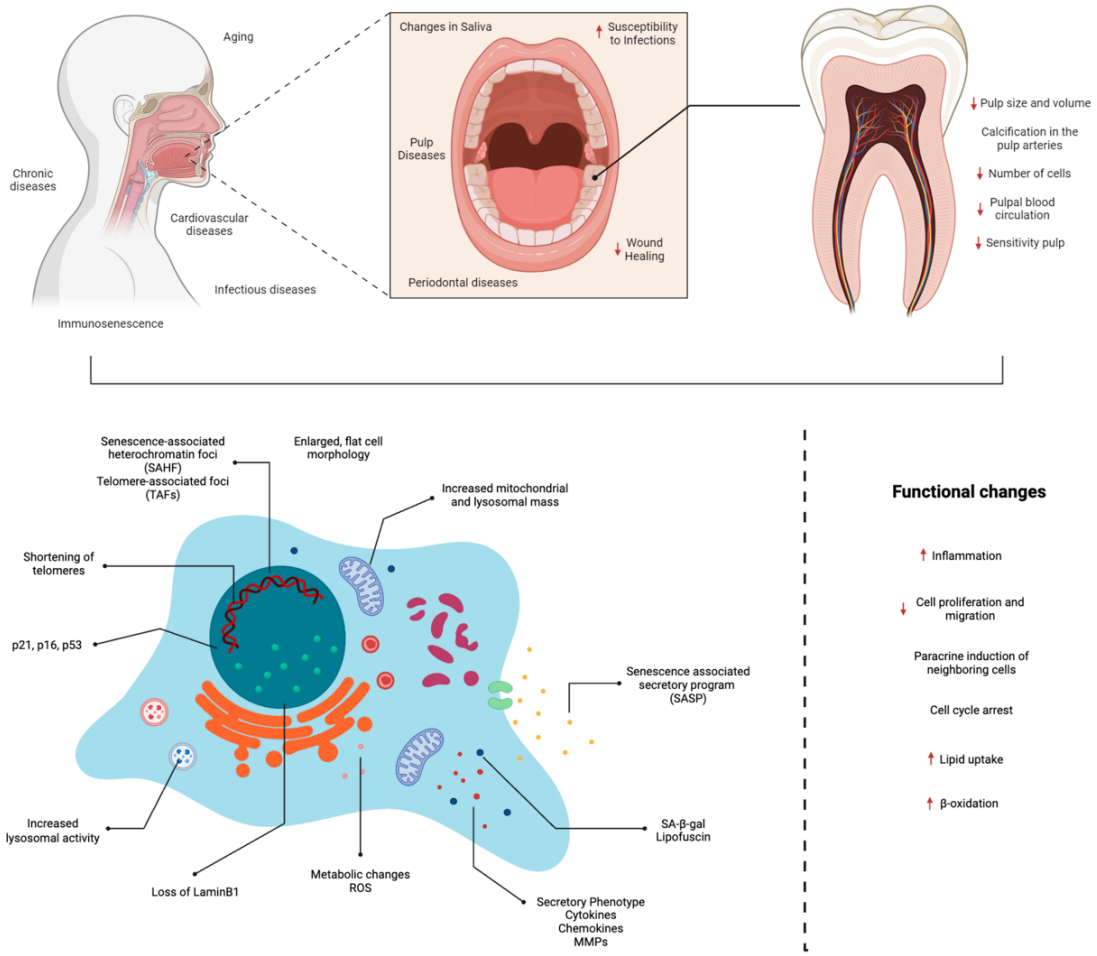

Figure 2. Schematic figure of the systemic repercussions of cellular senescence and its relationships with oral cavity and pulp. Created by BioRender.com.

IMPLICATIONS OF CELLULAR SENESCENCE IN DENTAL PULP

Over the years, the assessment of cellular senescence has become increasingly relevant, also in the context of dental pulp. Scientific advancements in the 21st century have led us further in the pursuit of understanding complex processes, such as senescence, including within dental research. The deepening of studies on the molecular and physiological changes resulting from this process has allowed for a more detailed understanding of the impacts of aging on pulp functionality (Farias et al. 2024).

A consistent increase in the annual number of publications, particularly after 2021, has been observed (Figure 3). Studies on cellular senescence in dental tissues

primarily focus on the dental pulp and periodontal ligament, while tissues such as the apical papilla and oral mucosa remain underexplored. Additionally, most articles focus on the diagnosis/pathogenesis of cellular senescence, while a lesser emphasis has been given to senescence treatment and prevention. Another important point observed was the predominance of cellular and molecular senescence studies, and a limited number of clinical investigations and systematic reviews. This highlights the need for more robust, evidence-based research. These findings reflect the current state of knowledge and emphasize significant gaps that warrant further exploration.

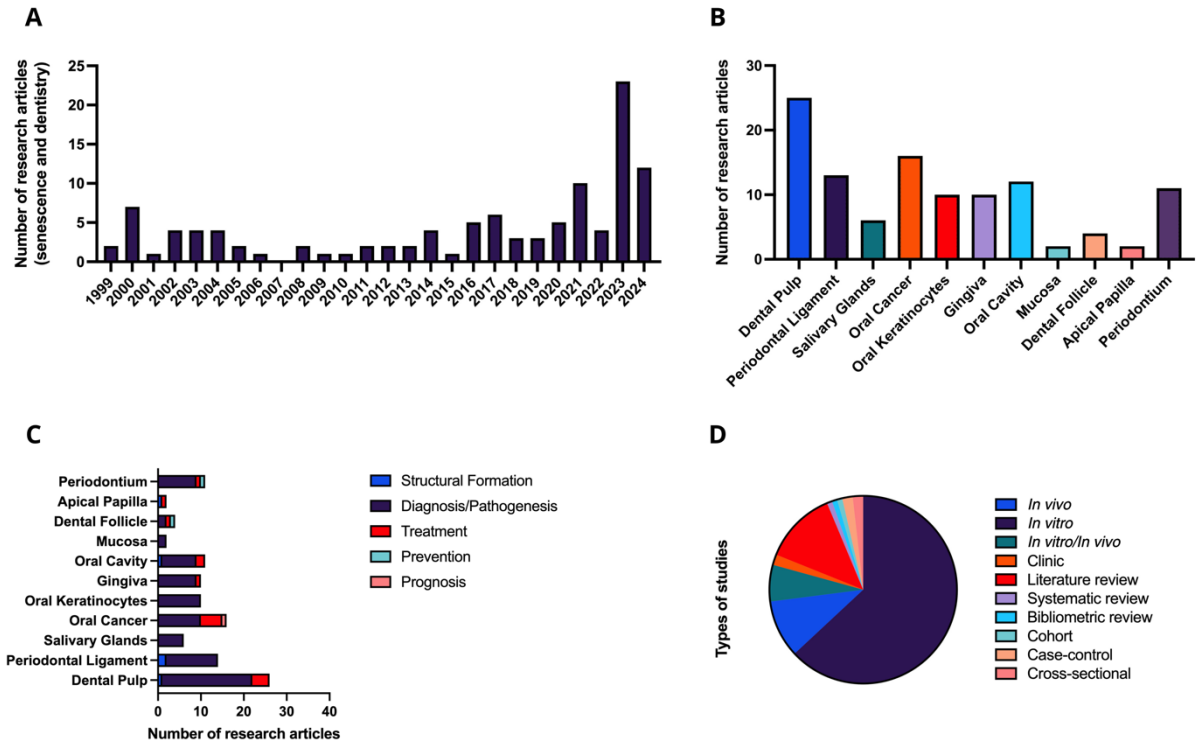

Figure 3. Overview of advances in dental research on cellular senescence over the last 25 years (1999-2024). A: Number of research articles published annually related to senescence and dentistry. B: Number of research articles published in each dental area of interest on cellular senescence. C: Number of research articles per area of interest and specific parameter analyzed. D: Assessment of the level of scientific evidence available on the topic, based on the types of studies published. Depending on the study focus, two or more parameters were counted.

The increasing relevance of cellular senescence in dental research, particularly regarding dental pulp, highlights a critical area of focus for understanding age-related changes in pulp functionality. The human dental pulp is a loose connective tissue

housed within dental elements, consisting of various cell types, including immune system cells, vascular cells, nerve cells, fibroblasts, odontoblasts, and stem cells. It is known that these cells perform numerous essential functions for maintaining dental homeostasis, such as formation, repair, nutrition, sensory, and immune response (Farias et al. 2023). However, aging can lead to the decline of these important pulp functionalities, which may include a decrease in pulp size and volume, calcification of the pulp arteries, reduction in the number of cells, decreased blood circulation, and sensitivity. Besides aging, other situations where the pulp may be exposed can also lead to changes related to cellular senescence, such as pulp stress caused by chronic caries, occlusal trauma, bruxism, dental cracks, and dental products that harm the pulp (Dzeletovic et al. 2020).

Regarding the decrease in pulp volume, it is known to occur due to the deposition of secondary dentin throughout life. Consequently, blood circulation may be interrupted in the dental pulp of older individuals. Additionally, dystrophic calcification may occur in the pulp arteries as the blood, lymphatic, and nerve supplies pass through the apical foramen and, as the apex narrows, these supplies may be interrupted. As for the decrease in the number of dental pulp cells, this occurs due to the reduced blood supply to the pulp cells (Maeda et al. 2020).

Concerning the physiological and molecular changes of this cellular state, it can be mentioned that senescent pulp cells exhibit increased inflammatory responses. This circumstance suggests a slower re-establishment of the inflammatory balance in senescent cells, which can affect the prognosis of conservative endodontic treatments, such as pulp capping (Galler et al. 2021). Additionally, the molecules related to odontoblasts present in the conglomerate of human pulp cells have an altered expression due to senescence. Thus, aging in pulp cells results in decreased function and physiological activity. Furthermore, odontoblasts of older individuals show signs of reduced autophagic activity, resulting in the accumulation of intracellular lipids and subsequent loss of functionality (Lee et al. 2024).

Moreover, pulp cells modify their morphology and acquire a flattened and fusiform shape. Other consequences of this cellular state include reductions in alkaline phosphatase activity, reduced proliferative potential, cellular differentiation, and neurogenic differentiation, lower resistance to apoptosis, and increased autophagic activity. Additionally, it affects the secretion of pro-inflammatory cytokines, which can

promote pulp mineralization, as well as telomere shortening and telomeric damage in senescent pulp cells (Iezzi et al., 2019). In addition, weak and chronic inflammatory conditions can trigger the induction of SASP (senescence-associated secretory phenotype) in pulp cells, causing senescence-related changes. Besides aging and stressful situations, several other factors have been reported in the literature as causes of cellular senescence, including diabetes and other pathologies and conditions (Asghari et al., 2021).

Furthermore, the implications of cellular senescence in dental pulp are significant and varied, potentially affecting several essential functions of this tissue. The consequences of this process are well-documented and of great interest to dentistry, particularly to endodontists in the context of pulp. Thus, the frequency and interaction among the main keywords related to senescence and dentistry was analyzed, highlighting the most recurring themes and their connections. A total of 1063 keywords were identified, with the most prevalent being "Cell" (320 occurrences), followed by "Senescence" (318 occurrences) and "Cellular Senescence" (206 occurrences). Figure 4 shows the most prevalent keywords (with 4 or more occurrences) and their collaborative relationships.

Based on research trends, terms like “Cell” and “Senescence” have increased significantly in recent decades, reflecting the growing awareness and exploration of cellular processes in dentistry. Common related research topics include “age,” “aging,” “oxidative stress,” and “DNA damage.” Additionally, researchers exploring “Cellular Senescence” frequently investigate queries such as “pulp tissue aging” and “inflammation”. Over the decades, the increase in occurrences of these keywords reflects a growth in interest and research into the role of cellular senescence in the dental pulp and in broader dental contexts.

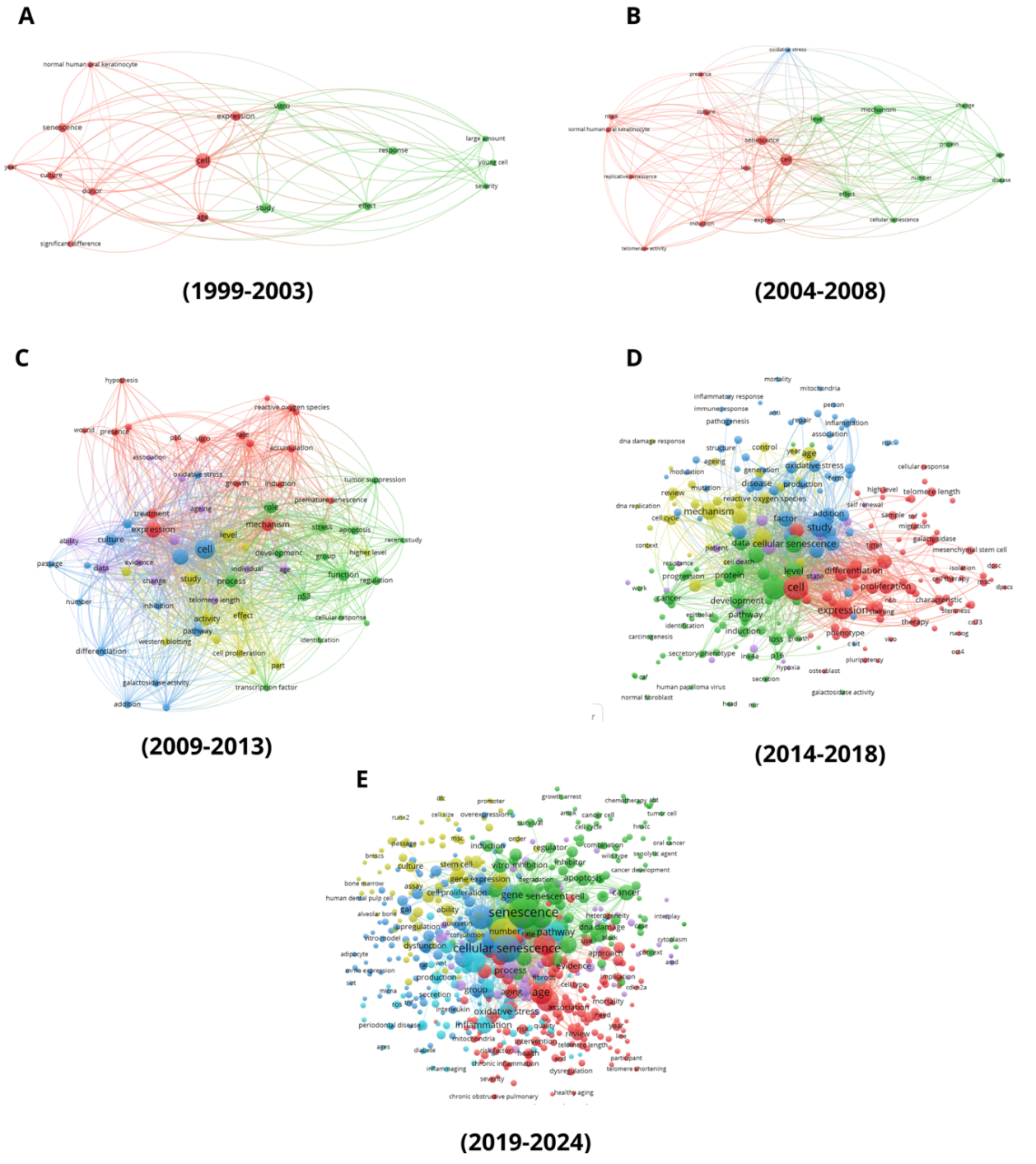

Figure 4. Analysis of the frequency and interaction of the main keywords associated with the study (1999-2024). A: publications between 1999-2003. B: publications between 2004-2008. C: publications between 2009-2013. D: publications between 2014-2018. E: 2019-2024. A minimum of 4 studies was established for the selection of keywords. The most recurring keywords are represented by larger points and in enlarged font, while the less frequent ones are associated with smaller points and presented in reduced font. The lines connecting the points illustrate the relationships and the shared use of these words in the studies considered. Created by VOSViewer.

Finally, to corroborate the findings of this research on the growing dental interest in cellular aging, a detailed citation analysis was conducted on the 50 most referenced articles from the past 25 years (1999–2024) related to senescence and dentistry. This analysis addresses the distribution and density of citations for each selected publication over the years, highlighting the development and increasing interest in research on cellular senescence in dental tissues. This bibliometric survey, therefore, provides a comprehensive view of research trends, leading authors, and approaches adopted over the last two decades, establishing a solid foundation for future investigations (Supplementary Table 1).

PRODUCTS FOR DENTIN-PULP COMPLEX USE THAT INDUCE SENESCENCE

Dentistry offers a wide range of products that aid in preventing, diagnosing, and treating various oral conditions. Among the most used products are restorative materials, desensitizing agents, and teeth-whitening products. It is well-known that cellular senescence can be caused by various factors, with exposure to stressors being one of them. The literature lacks sufficient research on this subject, which is likely to change over the coming decades as dentistry increasingly adopts a conservative and preventive approach. This shift focuses on the various repercussions of senescence in dental tissues (Table 1).

Despite the limitations in knowledge regarding the direct implications of using dental products on the dentin-pulp complex tissues leading to senescence, it is known that some of these products can be toxic to pulp tissue and other dental tissues. A clear example is dentin adhesive, which has the potential to penetrate through the dentinal tubules and reach the pulp, where it may exert a toxic effect. It has been found that certain dentin bonding agents, such as bisphenol-A glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA), present in adhesives, can inhibit cell proliferation. Additionally, hydrophilic monomers such as 2-hydroxyethyl methacrylate (HEMA) and TEGDMA, although less cytotoxic than their hydrophobic counterparts, can still influence the immune system, causing inflammatory responses (Sürmelioğlu et al., 2020).

HEMA, in particular, has been shown to diffuse through dentin and reach the pulp, with prolonged exposure to this agent potentially delaying cell migration, odontogenic differentiation, and mineralization. Additionally, high concentrations may inhibit the formation of reparative dentin, raising concerns about its safety in deep restorations (Bakopoulou et al., 2011). Furthermore, research suggests that Single Bond and HEMA, at different concentrations, can influence the expression and production of molecules related to inflammation. Therefore, the use of adhesive systems as pulp protection should be approached with caution due to their cytotoxic potential when in contact with pulp tissue (Modena et al., 2018).

Additionally, dentin adhesive agents release the photoinitiator camphorquinone (CQ), one of the photosensitizers responsible for generating free radicals, including reactive oxygen species, which have been associated with cytotoxic and mutagenic effects. Therefore, controlling the dose of these materials used and ensuring proper polymerization is crucial. However, further studies are needed to determine whether there is a direct correlation between these harmful effects and the factors that contribute to cellular senescence (Kazak et al. 2024).

Furthermore, restorative materials such as composite resins can release residual monomers either through degradation processes or incomplete polymerization of the resin materials, as well as other toxic components during and after the polymerization process. These released components have the potential to alter immune system cell responses or cause cellular damage, with possible genetic effects, such as the induction of genetic mutations or chromosomal aberrations. Such damage can lead to cellular stress, which, in turn, may promote cellular senescence (Carrillo-Cotto et al., 2020).

Additionally, dental whiteners, particularly those containing hydrogen peroxide, are widely used for teeth whitening. However, these agents are known to induce oxidative stress and damage to cellular DNA. When excessive amounts of peroxide reach the pulp chamber, there is a release of large volumes of inflammatory mediators, which triggers a significant inflammatory response that may potentially lead to cellular senescence. Moreover, prolonged exposure to whitening products containing hydrogen peroxide can result in increased levels of this compound in the pulp tissue, promoting lipid peroxidation, cellular membrane damage, and consequent cell death.

This process of cellular degradation releases free radicals that have the potential to induce genetic mutations and compromise cellular structural integrity (Torres et al. 2021).

Dental whitening protocols vary in terms of hydrogen peroxide concentration, application time, and activation methods, which can significantly influence their impact on pulp tissue. In-office treatments often use higher concentrations of hydrogen peroxide (30-40%), combined with light or heat activation, accelerating oxidative stress and increasing the likelihood of cellular damage. In contrast, at-home whitening protocols, which employ lower concentrations of peroxide (10-20%) over prolonged periods, can still contribute to the accumulation of oxidative stress. This chronic exposure is associated with cellular responses that induce the senescence-associated secretory phenotype (SASP), exacerbating inflammation and promoting tissue degradation. Consequently, the repeated application of whitening agents may create a microenvironment conducive to the induction of cellular senescence, compromising the function of dental pulp cells and impairing their regenerative capacity (Carey et al. 2015; Souza et al., 2021).

Product Type	Product name	Main components	Action on the Pulp Tissue	Impacts on Cellular Senescence	Reference
Oral antiseptic	Chlorhexidine (Biodinâmica, Ibiporã, Paraná, Brazil)	Chlorhexidine	Significant decrease in cellular activity, with L929 cell viability reduced to 30-39% after 1-minute and 10-minute treatments.	Reduction in cellular viability. Despite its antimicrobial effect, it may be toxic to dental pulp cells.	(Tsai et al. 2024)
Pulp capping	Calcium hydroxide cement (Dentsply, Charlotte, North Carolina, United States)	Calcium tungstate; zinc oxide; disalicylate ester of 1,3 butylene glycol and calcium phosphate	Clear cytotoxic effects detected, reducing survival of human pulp cells and inducing reactive oxygen species production.	Significant impact on cellular functions due to cytotoxicity and generation of reactive oxygen species (ROS) induction.	(Camargo et al. 2009)
Amalgam	Dental Amalgam (SDI, Bayswater, Australia)	Mixture of liquid mercury and metal alloy	Mercury induces different signaling pathways leading to cell death, DNA damage, and liver dysfunction.	Mercury release can induce oral damages since it can diffuse into the tooth pulp and gingiva. It also causes toxic effects in human gingival fibroblast.	(Reichl et al. 2006; Uçar and Brantley 2017; Shahi et al. 2019)
Glass ionomer	Riva Light Cure (SDI, Bayswater, Australia)	Powder: Radiopaque fluoroaluminosilicate glass, polymerization initiator and pigments Liquid: Polyacrilic acid, 2-hydroxyethyl methacrylate (HEMA), water, tartaric acid, stabilizers, and polymerization initiator	Mild to moderate pulp inflammation associated with slight tissue disorganization and deposition of reactionary tubular dentin.	Release of inflammatory mediators and possible cellular senescence.	(Ribeiro et al. 2020)
	Riva Self Cure (SDI, Bayswater, Australia)	Powder: Radiopaque fluroaluminosilicate glass, polyacrylic acid, pigments Liquid: Polyacrylic acid, tartaric acid, and water	Mild pulp inflammation associated with slight tissue disorganization.	Release of inflammatory mediators and possible cellular senescence.	(Ribeiro et al. 2020)
	RMGIC Vitrebond (3M, Maplewood, Minnesota, United States)	Dichlorodiphenyliodonium chloride, glass fiber	Known to release high levels of HEMA, which can cause persistent inflammation and adverse effects on pulp health.	High levels of HEMA released during setting may contribute to pulp damage and cellular senescence due to prolonged inflammatory response.	(Modena et al. 2009)
	RMGIC Vitremer (3M, Maplewood, Minnesota, United States)	Acrylic acid and acid copolymer, water, 2-hydroxyethyl methacrylate (HEMA) Ethyl acetate, Diphenyliodonium hexafluorophosphate	Monomers release may affect pulp vitality. High cytotoxicity observed for RMGICs may be due to unreacted resin monomers.	Similar concerns regarding monomer release impacting pulp health and cellular senescence.	Modena et al. 2009)

Acid	Phosphoric Acid (32%) (Bisco, Inc., Schaumburg, United States)	Phosphoric acid	Application on dentin removes smear layer, smear plugs as well as decalcifying peritubular and intertubular dentin.	Continuous outward dentin fluid movement, mainly in deep dentin, may cause rupture of primary odontoblast cells.	(Costa et al. 2000; Swift et al. 1995; Costa et al. 2003)
	Phosphoric acid (35%) (Bisco, Inc., Schaumburg, United States)	Phosphoric acid	Acid-etched deep dentin (remaining dentin thickness less than 300 μm) causes intense pulpal response.	Chronic irritation contributes to cellular senescence by inducing persistent inflammation and oxidative stress.	(Costa et al. 2002)
Adhesive	Adper™ Scotchbond™ 1 XT (3M, Maplewood, Minnesota, United States)	Phosphoric acid, bisphenol-A glycidyl methacrylate (Bis-GMA), HEMA	Moderate cytotoxicity, especially when applied to conditioned dentin. Potential diffusion through dentinal tubules can lead to pulp damage.	Alters collagen fibers in dentin and increases adhesive penetration, potentially raising cytotoxicity but with less impact than self-etch adhesives.	(Cardoso et al. 2021; Koulaouzidou et al. 2020; Caldas et al. 2020)
	Clearfil™ SE Bond 2 (Kuraray Noritake Dental Inc., Tokyo, Japan)	Hydroxyethyl methacrylate (HEMA), 10-MDP, dimethacrylate monomers, photoinitiators	Induces significant cytotoxicity, reducing metabolic activity and altering the cell cycle. Higher toxicity associated with its acidic pH and monomers, such as hydroxyethyl methacrylate (HEMA) and MDP which can further exacerbate these effects by creating an acidic environment and causing oxidative stress.	Increased cell death and cell cycle arrest, leading to reduced cellular functions and capacity for proliferation.	(Cardoso et al. 2021; Mantellini et al. 2003; Koliniotou-Koumpia and Tziafas 2005)
	Scotchbond™ Universal (3M, Maplewood, Minnesota, United States)	10-MDP, Bis-GMA, HEMA, ethanol, water	Low to moderate cytotoxicity, less aggressive compared to Clearfil SE Bond 2. Lower pH makes it less toxic overall but still has potential diffusion.	Reduces cellular proliferation and viability, but with less significant cell death compared to more acidic self-etch adhesives.	(Cardoso et al. 2021; Caldas et al. 2020)
Resin	Scotchbond MP (3M, Maplewood, Minnesota, United States)	Bisfenol A diglicidil éter dimetacrilato (BisGMA) and 2-hidroxietil metacrilato (HEMA)	Histological evaluation showed that material promoted at 7 days intense inflammatory response with predominance of neutrophils and macrophages.	Partially or unpolymerized resin particulates in contact with the connective tissue may induce persistent inflammatory response.	(Fantoni et al. 1992; Modena et al. 2009)
	Filtek Supremo (3M, Maplewood, Minnesota, United States)	Bis-GMA UDMA TEGDMA BisEMA, Zirconium and Silica	Release of TEGDMA and Bis-GMA monomers.	Release of toxic residual monomers towards the pulp that can impact the vascularization and repair process.	(Tabatabaee et al. 2008)

	Filtek Flow (3M, Maplewood, Minnesota, United States)	Bis-GMA UDMA TEGDMA BisEMA, Zirconium and Silica	Release of TEGDMA and Bis-GMA monomers.	Release of toxic residual monomers towards the pulp that can impact the vascularization and repair process.	(Tabatabaee et al. 2008)
	Bulk Fill (SDI, Bayswater, Australia)	SDR patented modified UDMA, TEGDMA, Barium and strontium alumino-floro-silicate glass	Included TEGDMA and UDMA concurrently. DMA and TEGDMA may be more toxic than other chemical compounds, potentially leading to reduced cell viability and a significant impact on human dental pulp stem cells (hDPSCs), which could compromise pulp health and regeneration.	ROS generation and glutathione depletion disrupt redox balance, leading to apoptosis. Monomers like TEGDMA, HEMA, and Bis-GMA increase ROS production in dental pulp cells.	(Gronthos et al. 2002; Chang et al. 2005; Chang et al. 2009; Eckhardt et al. 2009; Şişman et al. 2016)
	X-trafil (Voco, Cuxhaven, Germany)	Bis-GMA, UDMA, and TEGDMA	Included TEGDMA and UDMA concurrently. DMA and TEGDMA may be more toxic than other chemical compounds, potentially leading to reduced cell viability and a significant impact on human dental pulp stem cells (hDPSCs), which could compromise pulp health and regeneration.	ROS generation and glutathione depletion disrupt redox balance, leading to apoptosis. Monomers like TEGDMA, HEMA, and Bis-GMA increase ROS production in dental pulp cells.	(Gronthos et al. 2002; Chang et al. 2005; Chang et al. 2009; Eckhardt et al. 2009; Şişman et al. 2016)
Tooth whitening	Carbamide Peroxide and Hydrogen Peroxide Vital Bleaching (FGM, Joinville, Santa Catarina, Brazil)	10-15% Carbamide Peroxide or 2-10% Hydrogen Peroxide	Diffusion of hydrogen peroxide through dentin can lead to cytotoxic effects on the pulp, including inhibition of cellular enzymes like succinyl dehydrogenase, and damage to pulp tissue due to oxidative stress.	Chronic exposure to hydrogen peroxide induces oxidative stress, accelerating cellular senescence and reducing the reparative capacity of pulp cells.	(Hanks et al. 1993)

Table 1. Dental products that can cause pulp senescence.

PREVENTIVE STRATEGIES FOR CELLULAR SENESCENCE

During cellular senescence, cells exhibit a deficit in their capacity to divide in response to damage or stress stimuli, resulting in irreversible cell cycle arrest. Although this process plays a crucial role in preventing the accumulation of damaged cells, it contributes to tissue aging and the development of numerous chronic diseases (Farias et al., 2023). Recent research on cellular senescence has advanced significantly, highlighting the importance of pursuing methods to prevent and/or treat this state to maintain tissue health (Zonari et al., 2023).

Efforts to prevent and/or treat cellular senescence primarily focus on disrupting factors that trigger it, such as reducing oxidative stress. This stress arises from accumulating reactive oxygen species (ROS), which can damage DNA, proteins, and cellular lipids (Iakovou et al., 2022). Supplementation with antioxidants, such as vitamins C and E, and polyphenolic compounds in fruits and vegetables has been extensively studied. These antioxidants help neutralize ROS, protecting cells from oxidative damage (Ryan et al., 2010).

Additionally, strategies targeting telomeres and telomerase control are promising in cellular senescence. One of the leading causes of this process is telomere shortening, which leads to replicative senescence (Rossiello et al., 2022). Controlled activation of telomerase, the enzyme that maintains telomere length, is a potential approach that can delay cellular senescence in somatic cells. However, this strategy requires caution, as deregulated telomerase activation can increase oncogenesis risk, allowing indefinite cell proliferation with abnormally long telomeres (Xu et al., 2016).

Moreover, the modulation of cellular signaling pathways also represents a key area in preventing senescence (Mijit et al., 2020). The p53/p21 and p16INK4a/Rb pathways are essential in the cellular response to DNA damage, inducing senescence to prevent the division of damaged cells under stress conditions (Prieur et al., 2011). Studies indicate that resveratrol and rapamycin can modulate these pathways, reducing the risk of premature senescence (Bian et al., 2020).

Recent advancements also include the development of new analytic and xenomorphic drugs. Senolytics promote the selective elimination of senescent cells, while senomorphics modulate the phenotype of these cells, reducing their pro-

inflammatory activity without eliminating them (Zhang et al., 2023). Animal model studies show that senolytics like quercetin and dasatinib can decrease the burden of senescent cells in specific tissues and improve tissue function (Zoico et al., 2021; Novais et al., 2021). However, these approaches still require further studies to confirm their safety and efficacy in humans, but they represent a promising advancement in controlling cellular senescence.

Finally, specific peptides and growth factors have shown potential as therapeutic agents to prevent cellular senescence. Peptides such as GHK-Cu, FOXO4-DRI, OS-01, and UBX0101 have been investigated for their ability to modulate cellular pathways involved in senescence and promote tissue regeneration (Pickart et al., 2018; Baar et al., 2017; Zonari et al., 2024; Lane et al., 2021). Applying these peptides and growth factors in regenerative therapies is an emerging field aiming to enhance cellular and tissue function and slow the senescence process.

As research advances, these strategies have the potential to play a crucial role in regenerative medicine and the promotion of healthy aging, offering new possibilities for preventing diseases associated with the accumulation of senescent cells (Zonari et al., 2023). To better assess preventive strategies against cellular senescence, our work reviewed the literature on some promising drugs, as detailed (Table 2).

Product	Test phase	Mechanism of Action	Drug presentation	Target	Patent number	Reference
Azithromycin, roxithromycin and telithromycin	Preclinical	Selective elimination of senescent cells by inducing autophagy, aerobic glycolysis, and mitochondrial metabolic changes.	Oral tablets, injectable	Human fibroblasts, senescent cells	BR112021006423A2	Sotgia, Federica; Lisanti, Michael. Derivatives of azithromycin and roxithromycin as senolytic drugs. BR112021006423A2, 6 Jul. 2021.
Senolytic dental care product	Preclinical	Invention provides a method for tooth whitening as well as a method of treating periodontal disease, and/or for eliminating or reducing the number of senescent cells in the gingiva of a subject. Gingiva of a subject can thus be rejuvenated, potential loose teeth stabilized, and the well-being increased.	Dental care product: toothpaste, tooth gel, tooth mousse mouthwash, mouth spray and oral care foam	Tooth enamel	EUA11154470B1	Hug, Michael; Brönner, Haleh Abivardi; Signer, Golnar Abivardi; Lysek, Dominikus Amadeus. Anti-inflammatory and senolytic dental care product with tooth whitening characteristics. US11154470B1, 26 Oct. 2021.
Bcl-2 small molecule inhibitors	*In vivo* and *in vitro* studies. Venetoclax, a selective Bcl-2 inhibitor, was approved by the FDA in April 2016 for the treatment of chronic lymphocytic leukemia with 17-p deletion.	Bcl-2 family of proteins has also been found to be a potential target for the development of senolytic drugs, drugs that targeting senescent cells for the delay of aging or treatment of aging-associated disease.	Composition of the invention may comprise a pharmaceutically acceptable excipient, carrier or diluent	Senescent cells	US2019054097A1	Zhou, Daohong; Zheng, Guangrong; Zhang, Xuan; Wang, Yingying; Chang, Jianhui; Xia, Fen; De Almeida, Maria Schuller; Kim, Ha-neui; Zhang, Peiyi. Compositions targeting senescent cells and the uses thereof. US2019054097A1, 21 Feb. 2019.

Quercetin	Preclinical	Senolytic agent is an antibody, aptamer, targeted ubiquination construct, or other biological molecule, for example, comprising a peptide or nucleic acid, which selectively binds to antigens present on senescent and wherein such binding mediates, directly or indirectly, the death or elimination of such senescent cell or effector of senescence.	Molecular Capsule	Senescent cells	EUA20200078376A1	Brushan, Anil. Inhibiting senescent processes in beta cells for the prevention of type 1 diabetes. US20200078376A1, 12 Mar. 2020.
Dasatinib + Quercetin	Clinical	Selective death of cells that have acquired a senescent phenotype.	Combined Compound	Senescent cells	EUA20200078376A1	Bhushan, Anil. Inhibiting senescent processes in beta cells for the prevention of type 1 diabetes. US20200078376A1, 12 Mar. 2020
Milk Thistle Flower Extract	Preclinical	Selectively induces apoptosis in senescent cells, restores youthful cell morphology and function.	Standardized extract derived from Silybum marianum flowers	Senescent cells exhibiting the senescence-associated secretory phenotype (SASP)	KR102451009B1	우지은; 신승우; 정은선; 박덕훈; 마틸드, 프레셰; 하난 샤크라. Composition for preventing or treating cell senescence associated diseases comprising Silybum marianum flower extract. KR102451009B1, 6 Oct. 2022.
Homoharringtonine-based senotherapeutic composition	Preclinical	Senolytic effect selectively targeting senescent cells to induce apoptosis, reducing aging-associated fibroblasts and renal cells while preserving or restoring healthy cells.	Composition containing homoharringtonine as active ingredient for preventing or treating cellular senescence-associated diseases	Senescent cells associated with diseases like cancer, diabetes, cardiovascular and neurological degeneration, obesity, and tissue fibrosis	US11992492B2	Kim, Jae-Ryong; Kim, Eok-Cheon; Jung, Kyong-Jin; Bin, Bum-Ho; Son, You Lim. Composition for preventing or treating cellular senescence-associated diseases, containing homoharringtonine as active ingredient. US11992492B2, 28 May 2024.
Fermented Barley Seed Extract	Preclinical	Product qualifies as a senolytic agent, which is aimed at eliminating senescent cells.	Fermented barley seed extract to be usefully used in cosmetics and pharmaceutical compositions	Extract targets various aging-related diseases by specifically influencing	KR20230087265A	Woo, Ji Eun; Shin, Seoung Woo; Sim, Junbo; Jung, Eun Sun; Park, Deok Hoon. Composition for preventing or treating cell senescence associated diseases comprising fermented

				senescent cells, leading to improved health outcomes		barley seeds extract. KR20230087265A, 16 Jun. 2023.
GSTO1-Inhibitor	Pre-clinical/Clinical Trials	Selective senolytic activity targeting senescent cells to reduce chronic inflammation and promote tissue regeneration.	Does not specify	GSTO1 protein	CN115475155A	Zhu, Yinhua; Wang, Jigang; Tang, Huan; Ma, Ang; Zhang, Ying; Zhang, Qianyu; Chu, Zheng; Zhang, Ying; Wang, Chen; Gao, Peng; Xia, Fei; Guo, Qiuyan; Shi, Qiaoli. Application of GSTO1 protein small-molecule inhibitor in anti-aging and treatment of aging-related diseases. CN115475155A, 16 Dec. 2022.
Lysosome-targeted senolytics prodrug (Lyso-Sphβ-gal)	*In vitro*/ *In vivo* testing	Prodrug accumulates in the lysosomes of senescent cells, where it is activated by β-galactosidase, releasing sphingosine and inducing apoptosis.	Prodrug	Lysosomes of senescent cells	CN114249782A	Liu, Yanlan; Xia, Yinghao; Li, Jili; Wang, Linlin; Luo, Qianyuan; Xie, Yuqi. Prodrug for selectively destroying senescent cell lysosome as well as preparation method and application of prodrug. CN114249782A, 29 Mar. 2022.
Ginkgo biloba extract	Preclinical/Clinical	Targets and induces senescent cells in the tumor microenvironment.	Oral, injectable, or topical formulations	Senescent cells in tumor microenvironment, particularly those resistant to chemotherapy	WO2022166839A1	Sun, Yu; XU, Qixia; Zhang, Xuguang; He, Ruikun. Use of Ginkgo leaf extract in preparation of drug for targeting senescent cells, inhibiting tumors or prolonging lifetime. WO2022166839A1, 11 Aug. 2022.
Salvianolic Acid B (SAB)	Preclinical	Inhibit the expression of senescence-associated secretory phenotype (SASP), anti-aging or removing senescent cells, reduce tumor resistance to chemotherapy drugs; and/or extend lifespan or survival in old age.	Oral tablet, injectable	Senescent cells; SASP factors	CN117205197A	Sun, Yu. Application of salvianolic acid B (SAB) as novel anti-aging drug raw material in cell aging, tumor treatment and life prolonging. CN117205197A, December 12, 2023.
Cocoa Extract + Mitoxantrone	Preclinical	Induces apoptosis in senescent tumor cells; inhibits SASP.	Injectable = Weight ratio: Mitoxantrone to Cocoa Extract 1:20 to 80; preferred 1:40-60	Prostate, breast, lung, and other cancers	CN112870238A	Sun, Yu; Ding, Zhengbing. Application of cocoa extract in preparation of medicine for resisting aging and inhibiting tumors. CN112870238A, June 1, 2021.

Cocoa Extract + Bleomycin	Preclinical	Inhibits SASP expression and promotes cell death in senescent cells.	Injectable = Low concentration: Bleomycin 30-70 μg/mL, Cocoa Extract 80-120 μM (preferred 90-110 μM), or High concentration: Bleomycin 30-70 μg/mL, Cocoa Extract 150-2000 μM (preferred 200-1200 μM)	Tumor cells producing SASP	CN112870238A	Sun, Yu; Ding, Zhengbing. Application of cocoa extract in preparation of medicine for resisting aging and inhibiting tumors. CN112870238A, June 1, 2021.
Cocoa Extract + Doxorubicin	Preclinical	Targets senescent cells in tumor microenvironment; induces cell apoptosis.	Injectable = Weight ratio: Doxorubicin to Cocoa Extract 1:4 to 16; preferred 1:8-12. Final concentration of Doxorubicin: 20-60 μg/mL (preferred 30-50 μg/mL). Cocoa Extract final concentration: 80-120 μM or 150-2000 μM	Drug-resistant tumors	CN112870238A	Sun, Yu; Ding, Zhengbing. Application of cocoa extract in preparation of medicine for resisting aging and inhibiting tumors. CN112870238A, June 1, 2021.
Vine tea extract mixed with chemotherapeutic drugs	Preclinical	Targets senescent cells, enhancing chemotherapy effects and inhibiting tumor growth.	Pharmaceutical composition or a kit for inhibiting tumors	Tumors (prostate, breast, lung, etc.) and tumor microenvironment	WO2022166841A1	Sun, Yu; Xu, Qixia; Zhang, Xuguang; He, Ruikun. Use of rattan tea extract in preparation of drugs targeting senescent cells, inhibiting tumors, or prolonging life. WO2022166841A1, Aug. 11, 2022.

Table 2. Promising drugs for preventive strategies against cellular senescence.

CONCLUSION

This review provided an in-depth analysis of the literature on the relationship between cellular senescence and oral health, highlighting the importance of this biological phenomenon in aging and associated oral pathologies. Dental research has intensified in the search to understand the mechanisms related to cellular senescence, given its impact on the prognosis of clinical treatments, such as conservative endodontic procedures. The analysis of scientific production revealed a substantial increase in publications on cellular senescence in dental tissues, particularly after 2021, with an emphasis on the dental pulp and periodontal ligament. However, most studies focus on the pathogenesis and diagnosis of cellular senescence, with a significant gap in therapeutic and preventive approaches. The predominance of cellular and molecular studies, as opposed to clinical investigations and systematic reviews, points to the need for more robust, evidence-based research. This situation underscores the urgency of further investigating the effects of dental materials, such as dentin adhesives, composite resins, and tooth whiteners, which release toxic substances that can induce cellular stress, inflammation, and genetic damage, contributing to cellular senescence in dental tissues. While dentistry is gradually adopting more conservative and preventive approaches, a deeper understanding of the effects of these materials on cellular senescence still requires further investigation. Additionally, therapeutic strategies involving senolytic and senomorphic drugs have emerged as promising approaches, demonstrating great potential for effectively modulating cellular senescence.

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SUPPLEMENTARY INFORMATION

Title of the article	Citation count (Web of Science)	Citation count (Elsevier Scopus)	Citation count (Dimensions)	Citation count (Google Scholar)	Citation density
(1) Murray, P. E., Stanley, H. R., Matthews, J. B., Sloan, A. J., & Smith, A. J. (2002). Age-related odontometric changes of human teeth. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 93(4), 474–482.	159	189	192	333	9,9
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Supplementary table 1. Citation analysis of the top 50 most cited articles related to senescence and dentistry over the years (1999-2024).

CAPÍTULO 2 (Intended submission: Journal of Endodontics; IF = 3.5)

Impact of Cellular Senescence on the Immune-inflammatory Response and Regenerative Capacity of Human Dental Pulp Cells

ABSTRACT

Introduction: Cellular senescence is a state of permanent cell cycle arrest that, although it plays a protective role, can compromise tissue homeostasis due to the secretion of inflammatory mediators and alterations in the cellular microenvironment. In the dental pulp, this process can reduce repair capacity and increase susceptibility to inflammation and tissue degeneration. Additionally, senescence can affect the immune response, contributing to a persistent inflammatory environment. However, its impact on the immune response of pulp cells has not yet been fully elucidated. This study aims to evaluate the effects of senescence on the viability, morphology, migration, proliferation, and immune response of human pulp cells. **Methods:** Pulp cells were cultured from healthy third molars using the explant technique. Cellular senescence was induced with doxorubicin (500 μM), and the senescent state was confirmed by β-galactosidase staining. Cells were exposed to LPS and LPS + IFN-γ to simulate immunoinflammatory conditions. Morphological analysis was performed by scanning electron microscopy; cell viability and proliferation were assessed using Trypan Blue; and migration was measured by the "scratch" method. The expression of inflammatory and anti-inflammatory mediators (IDO, TNF-α, IL-6, IL-10, and TGF-β1) was evaluated by qPCR. **Results:** The results of this study show that cellular senescence compromises essential functions of the dental pulp, negatively impacting its regenerative and repair capacity, especially under inflammatory conditions. Senescent pulp cells showed reduced viability, proliferation, and migration, as well as morphological changes indicative of decreased tissue function. Inflammatory pathways were also modulated, with an increase in the expression of pro-inflammatory cytokines, such as IDO, TNF-α, and IL-6; no increase in IL-10 expression even under inflammatory stimulus; and a decrease in the expression of regenerative cytokines, such as TGF-β1. **Conclusions:** The findings suggest that senescence can directly impact the

prognosis of conservative endodontic treatments, especially in patients with pulp compromised by lesions. This highlights the need for therapeutic strategies that mitigate the effects of senescence in order to preserve pulp vitality.

Keywords: Dental Pulp; Senescence; Aging; Inflammation; SA-β-Gal; Conservative endodontic treatments.

INTRODUCTION

Cellular senescence is a state of permanent cell cycle arrest, usually triggered by factors such as telomere shortening and cellular stressors (1). Although this mechanism plays a protective role by controlling the proliferation of damaged cells, it is known that the accumulation of senescent cells can lead to tissue dysfunction due to the secretion of inflammatory mediators and changes in the cellular microenvironment (1). This phenomenon, called the senescence-associated secretory phenotype (SASP), can contribute to chronic inflammatory and degenerative processes (2). In the dental pulp, this process can directly influence tissue homeostasis and the response to injuries.

In general, this state contributes to aging of dental pulp, to age-related diseases and to decline in cellular functions in this tissue. In addition, senescence can also affect the pulpal immune response, through a process known as immunosenescence, characterized by progressive changes in the immune system that compromise the innate and adaptive immune response (3). In parallel, senescence is also accompanied by a state of low-grade chronic inflammation related to aging, known as inflammaging, which may contribute to the persistence of an inflammatory environment that is detrimental to cellular homeostasis (2).

In addition, senescent cells also secrete a variety of molecules, including cytokines, chemokines, proteases, and growth factors, which influence the behavior of neighboring cells, inducing a state of senescence in them or modifying their functions (4). This phenomenon occurs through paracrine signaling, a type of cellular communication in which secretory cells release

substances into the local microenvironment, affecting nearby cells without requiring direct contact. Thus, the presence of senescent cells in the pulp not only exhibit impairment of their functions, but also contribute to the spread of senescence in healthy cells, exacerbating the inflammatory process and compromising tissue regeneration (5, 3).

The dental pulp, a specialized connective tissue essential for maintaining tooth vitality, becomes progressively more susceptible to cellular senescence with advancing age or exposure to deleterious stimuli. In our previous study, we observed that senescent pulp cells exhibit morphological changes, reduced proliferative and migratory capacities, in addition to an increase in the secretion of inflammatory mediators (6). These changes compromise the capacity for tissue repair and make the pulp more susceptible to inflammatory and degenerative processes (1).

Furthermore, the immune response of pulp cells is a determining factor in maintaining tissue homeostasis and the ability to respond to injuries (1). However, the impact of senescence on this response has not yet been fully elucidated. Studies suggest that senescent cells can negatively influence the pulp microenvironment, contributing to the persistence of inflammation and compromising cell repair and defense mechanisms (7, 8).

Therefore, this study aims to evaluate the immune response of senescent and non-senescent pulp cells under different inflammatory conditions. Understanding these mechanisms is essential to improve the prognosis and possibilities of conservative endodontic treatments. Furthermore, our findings may contribute to the development of new therapeutic approaches for dentistry, aiming at strategies for modulating this process, which should contribute to the preservation of pulp vitality.

METHODOLOGY

Primary Culture of Dental Pulp Cells

Pulp tissues were obtained from intact third molars extracted from donors aged between 18 and 30 years, after approval by the University of Brasília ethics committee (CAAE: 75393923.1.0000.0030). In a laminar flow hood, teeth were washed with phosphate-buffered saline (PBS) and fractured using orthodontic pliers (Quinelato, Rio Claro, São Paulo, Brazil). Coronal and radicular dental pulp was carefully removed using manual K-type endodontic files (Dentsply Sirona, Charlotte, North Carolina, USA). Dental pulps were washed with PBS containing 100 $U.mL^{-1}$ of penicillin (Invitrogen, Grand Island, New York, USA), 100 $\mu g.mL^{-1}$ of streptomycin (Invitrogen), and 1% amphotericin B (Invitrogen). Then, pulp tissue was minced with a scalpel blade and fixed in 6-well cell culture plates (Kasvi, São José dos Pinhais, Paraná, Brazil). Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, San Luis, Missouri, USA) supplemented with 20% fetal bovine serum (FBS) (GIBCO, Grand Island, New York, USA), 100 $\mu g.mL^{-1}$ penicillin (Invitrogen), 100 $\mu g.mL^{-1}$ streptomycin (Invitrogen), and 2 $\mu g.mL^{-1}$ glutamine (GIBCO) was added to each well. Plates were maintained in a humidified atmosphere containing 5% CO_2 at a temperature of 37 °C. Cells were subcultured as they reached confluence to obtain enough cells for each experiment (9).

Cellular Senescence Induction and Immunoinflammatory-tested-conditions

Cellular senescence was *in vitro* induced by doxorubicin (Doxorubicin hydrochloride, Sigma Aldrich). For this, pulp cells were incubated with 500 µM of doxorubicin (Sigma Aldrich) in DMEM medium (Sigma Aldrich) supplemented (as previously described) for 24 h. Then, medium was changed to supplemented DMEM (Sigma Aldrich) medium with 10% fetal bovine serum (FBS) (GIBCO), with subsequent medium changes every 72 h, for 7 days. Non-senescent group was represented by cells incubated with supplemented DMEM medium (Sigma Aldrich). After cellular senescence protocol, cells were washed twice for 30 seconds with PBS. Immediately after that, cells were fixed with a fixing solution

[2% formaldehyde (Dinâmica, Indaiatuba, São Paulo, Brazil) and 0.5% glutaraldehyde (Dinâmica)], for 5 minutes at room temperature and then washed again with PBS solution. Subsequently, 1 mL of X-Gal staining solution [40 mM citric acid solution (Dinâmica) and sodium phosphate (Sigma Aldrich), 5 mM potassium hexacyanoferrate (Sigma Aldrich), 150 mM sodium chloride (Sigma Aldrich), 2 mM magnesium chloride (Sigma Aldrich), 1 $mg.mL^{-1}$ X-Gal (Thermo Fisher Scientific, Waltham, Massachusetts, USA)] was added and the plate was incubated at 37 °C protected from light, overnight. After 16 h, cells were washed twice for 30 seconds with PBS and once with methanol (Dinâmica). Then, cells were observed and photographed in the central region of each well, always with the same magnification (4X), using an inverted light microscope with phase contrast Axio Observer D1 (Zeiss, Oberkochen, Germany). Stained and unstained cells were counted using Image J software (NIH, Bethesda, Maryland, USA) (10).

To simulate different *in vitro* immunoinflammatory conditions, LPS (1 $\mu g.mL^{-1}$) (lipopolysaccharides from Escherichia coli, Sigma) and IFN-γ (1 $\mu g.mL^{-1}$) (Interferon-gamma, Sigma) were added as stimuli, either individually or combined, for 24 hours (11).

Scanning Electron Microscopy Assay

The morphological changes were analyzed through Scanning Electron Microscopy (SEM). Non-senescent and senescent pulp cells (2 x 10^5 cells per well) stimulated with different immunoinflammatory conditions were seeded in 6-well culture plates (Kasvi) with DMEM medium (Sigma Aldrich) supplemented (as previously described). Round glass coverslips of 13 mm × 13 mm (Fisher Scientific, Suwanee, Georgia, USA) were placed at the bottom of the plates before cell cultivation. Coverslips were fixed in Karnovsky's solution 0.1M (2% glutaraldehyde and paraformaldehyde) for 24 hours, followed by two washes with 0.1M sodium cacodylate solution. Then, cells were then fixed in 1% osmium tetroxide for 30 minutes and washed twice with distilled water. Dehydration was performed in acetone solutions at 50%, 70%, 90%, and 100%. After drying the glass coverslips, they were metallized and analyzed using a scanning electron

microscope (JSM 7001F, Jeol). Images were captured at magnifications of 1500x, 3000x, and 5000x. For the analysis, areas containing at least 10 cells were selected, ensuring greater statistical robustness. The quantitative analysis was performed regarding cell size and the number of cell extensions using ImageJ software (NIH, Bethesda, MD) (Fig. 2A) (12).

Cell Migration Assay

Cell migration was performed using the scratch method. Non-senescent and senescent pulp cells (2.5 x 10^5 cells per well) stimulated with different immunoinflammatory conditions were seeded in 6-well culture plates (Kasvi) with DMEM medium (Sigma Aldrich) supplemented (as previously described). Cells were maintained until a confluent monolayer was formed. A central well wound was removed using a 1000 µL micropipette tip with a large opening. Next, culture medium was changed to DMEM (Sigma Aldrich) without FBS (GIBCO). Cultures were incubated and monitored for up to 48 h. Photographs were taken using microscopy at 0, 24, and 48 h for later analysis (13). Images were processed, and cells in the wound area were counted using Image J software (NIH).

Cell Viability and Proliferation

Senescent and non-senescent pulp cells ($2x10^4$ cells) stimulated with different immunoinflammatory conditions were cultured in DMEM medium (Sigma Aldrich) without FBS (GIBCO), in 96-well plates (Kasvi). After incubation period, cells were resuspended, and a 0.4% Trypan Blue staining solution (Sigma Aldrich) was added for 1 minute. Live and dead cells were immediately counted at 24 and 48 h (14).

Production of Inflammatory and Anti-inflammatory Mediators

The expression of *IDO, TNF-α, IL-6, IL-10,* and *TGF-β1* by senescent and non-senescent pulp cells was evaluated using real-time qPCR. The senescent and non-senescent cell groups were assessed under different

immunoinflammatory conditions, as well as in basal conditions (unstimulated cultures). After cell culture, RNA was extracted using the TRIzol™ method (ThermoFisher Scientific, California, USA) (5). Then, RNA quantification was determined by Qubit® (6). Gene expression analysis was performed by real-time qPCR using the StepOnePlus™ Real-Time PCR System (ThermoFisher) to verify the expression of genes indicative of inflammatory and anti-inflammatory processes. For this analysis, the gene expression was evaluated. GAPDH was used as the housekeeping gene. Quantification was performed according to the manufacturer's recommendations. Each reaction had a final volume of 10 µL, consisting of: 5 µL of SYBR Green; 0.5 µL of primers, with 0.25 µL of primer for each gene (Table 1); and 2.5 µL of nuclease-free water (ThermoFisher Scientific - SYBR™ Green PCR Master Mix KIT). The relative expression levels in the experimental group were calculated using the Δ(ΔCT)(ΔCTControl − ΔCTExperiment) method (15).

Gene	Forward	Reverse
GAPDH	TCAACGACCACTTTGTCAAGCTCAGCT	GGTGGTCCAGGGGTCTTAC
IDO	GGGAAGCTTATGACGCCTGT	CTGGCTTGCAGGAATCAGGA
TNF-α	CACAGTGAAGTGCTGGCAAC	GATCAAAGCTGTAGGCCCCA
IL-6	TCAATATTAGAGTCTCAACCCCCA	TTCTCTTTCGTTCCCGGTGG
IL-10	GGTGGTCCAGGGGTCTTAC	ACTCTGCTGAAGGCATCTCG
TGF-β1	GCTGTATTTAAGGACACCGTGC	TGACACAGAGATCCGCAGTC

Table 1. Primers Sequence for Each Gene Used in the PCR Assay. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); Indoleamine-pyrrole 2,3-dioxygenase (IDO); Tumor necrosis factor alpha (TNF-α); Interleukin-6 (IL-6), Interleukin-10 (IL-10); Transforming growth factor beta 1 (TGF-β1).*

Statistical Analysis

All experiments were carried out in technical and biological triplicates. Data were described as means and standard deviations. The normality of the data was assessed using the Shapiro-Wilk test, and the appropriate statistical analysis was applied accordingly. Senescence induction was statistically analyzed by

Student's t-test, while cellular migration, proliferation, viability, and immunomodulation was analyzed by one-way ANOVA. All analyses were performed using Graph Pad Prism 10 software and considered at a significance level of 95%.

RESULTS

Senescence induction protocol and effect of senescence on pulp cell viability under immunoinflammatory conditions

Pulp cells' senescence was confirmed by the β-galactosidase staining assay, where 99% of cells exposed to doxorubicin showed positive staining, in contrast to the non-senescent group (***$p < 0.0002$, Figure 1B, C, and D). Thus, this protocol was used for subsequent assays. Senescent pulp cells' viability was statistically lower after 24 h and 48 h of exposure to immune-inflammatory conditions (LPS and LPS plus IFN-γ). The viability of senescent pulp cells was significantly decreased after 24 and 48 hours of exposure to immune-inflammatory conditions (LPS and LPS plus IFN-γ), with the lowest viability observed at approximately 70% under LPS plus IFN-γ condition. These findings suggest that senescent cells exhibit increased vulnerability to viability loss when subjected to inflammatory stimuli, indicating greater susceptibility in inflammatory microenvironments (*$p < 0.05$, Figure 1E, F, G and H).

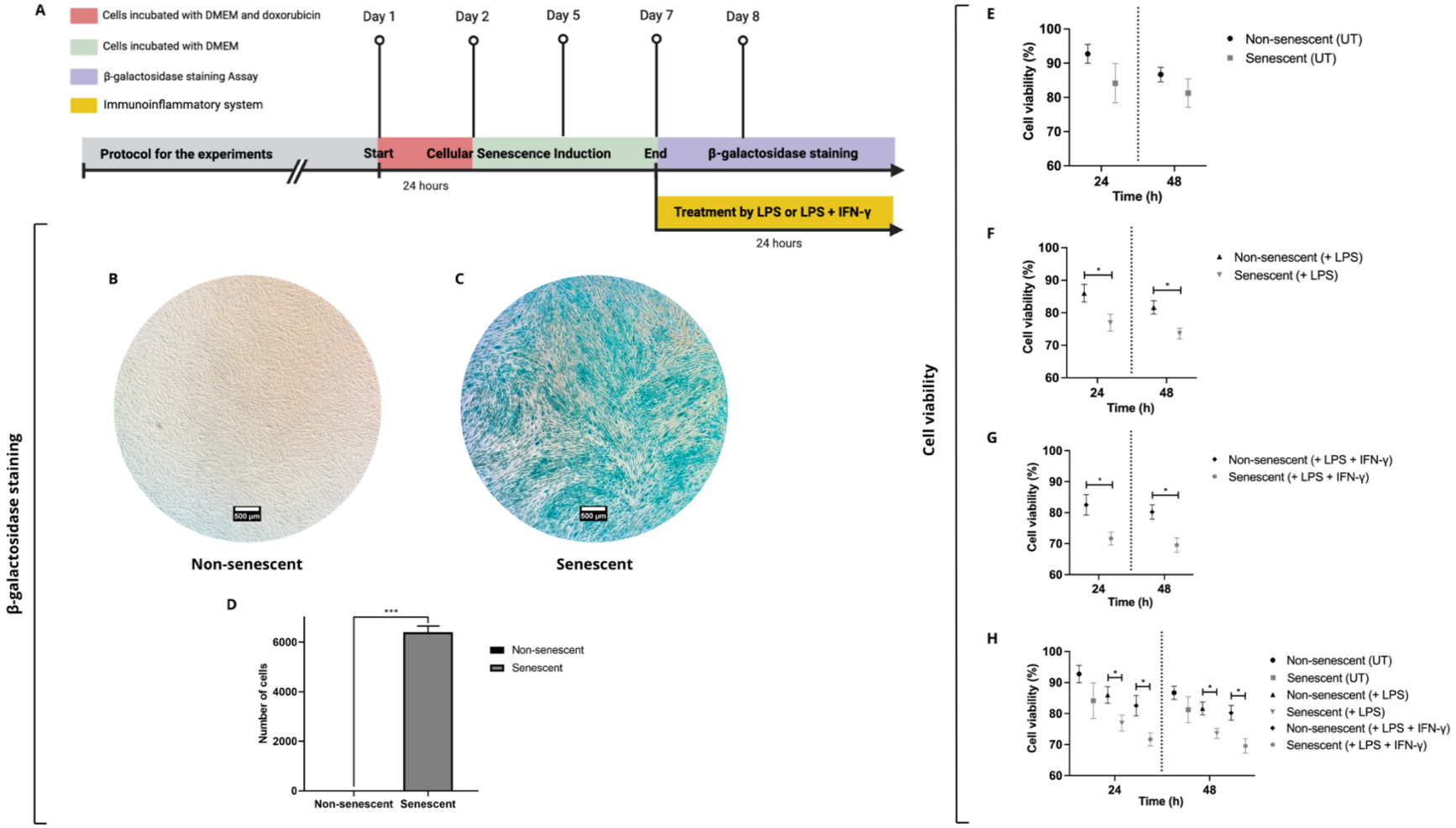

Figure 1. Human dental pulp cells after β-galactosidase staining assay and their viability under different conditions. (A) Schematic diagram of induced senescence protocol and its confirmation by β-galactosidase staining assay, along with the in vitro immune-inflammatory system model. Non-senescent (B) and senescent (C) cells. Scale bar: 500 µm. Number of senescent human dental pulp cells after β-galactosidase staining (D). ***$p < 0.0002$, by Student's t-test. Viability of non-senescent and senescent primary pulp cells in untreated (UT) culture (E), in LPS-stimulated culture (F), in LPS plus IFN-γ-stimulated culture (G), and comparative analysis between all assay groups (H). *$p < 0.05$, by one-way ANOVA test.

Effect of senescence on pulp cells morphology in immunoinflammatory conditions

Microphotographs obtained at 1500x, 3000x, and 5000x magnifications (Fig. 2A) by SEM were analyzed to assess the morphological differences between senescent and non-senescent cells, considering two main parameters: cell size and the number of cell extensions. A marked increase in the size of senescent cells was observed ($P < 0.0001$; Figure 2B, C and D), possibly related to alterations in the cytoskeleton and changes in intracellular organization associated with the senescent state. These morphological changes were evident both under homeostatic conditions, represented by the untreated group, and in inflammatory conditions induced by LPS and LPS plus IFN-γ, suggesting that cellular senescence influences cell structure regardless of the inflammatory stimulus. Additionally, a significant reduction in the number of cell extensions in senescent cells compared to non-senescent cells in the different treatment groups ($P < 0.0001$; Figure 2E and F) was found, suggesting a reduced capacity for cell interaction and communication.

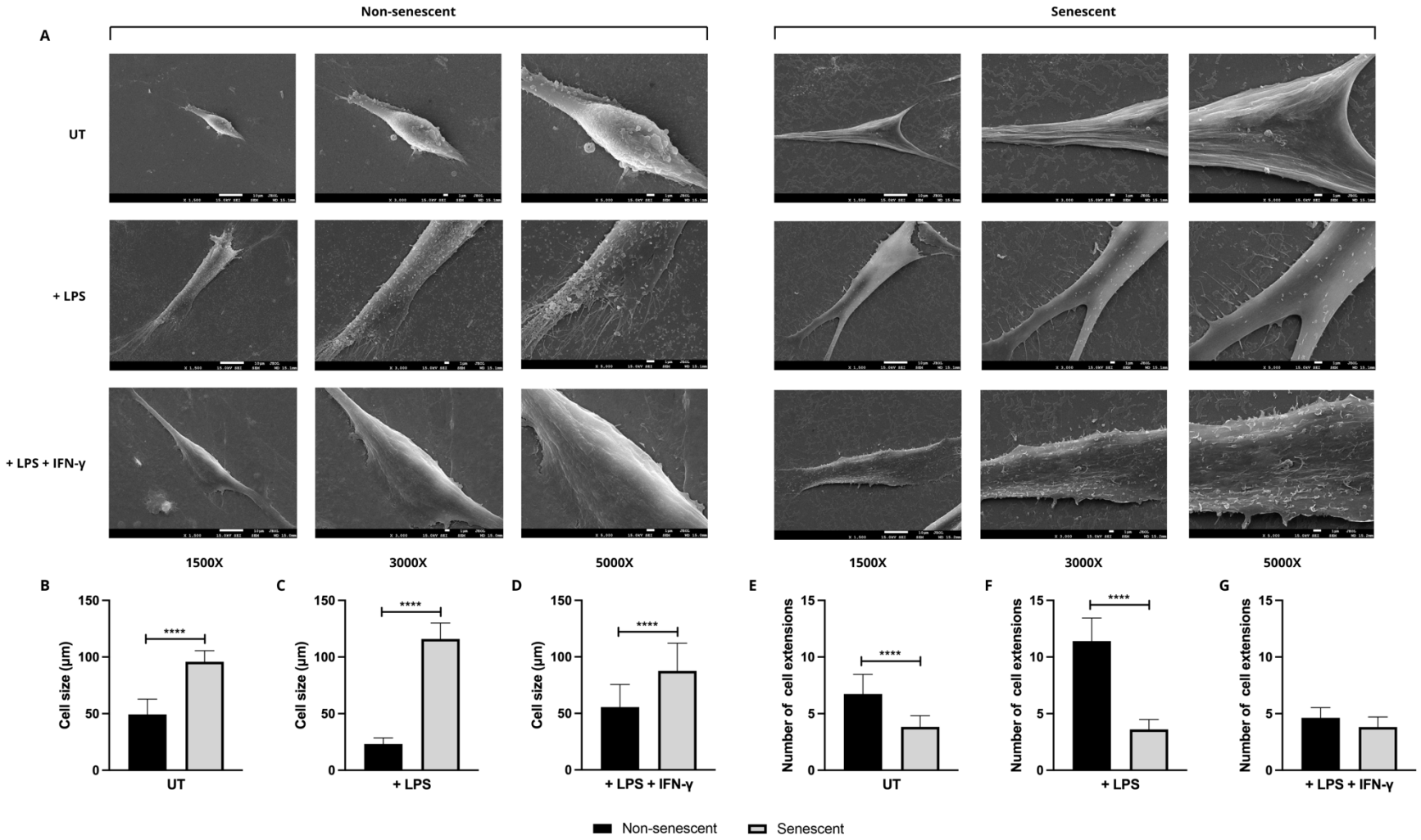

Figure 2. Morphological analysis of senescent and non-senescent pulp cells under different conditions. Dental pulp cells were photographed using SEM at magnifications of 1500x, 3000x, and 5000x, as shown in the columns. Each row represents a different cell condition: untreated, treated with LPS, and treated with LPS plus IFN-γ (A). The quantitative analysis of the images obtained by SEM compared the senescent group to the non-senescent group under different treatment conditions, considering the number of cell extensions and cell size. Analyses for each treatment condition are presented for the untreated group (UT) (B, E), for the group in the presence of LPS (C, F), and for the group stimulated by LPS plus IFN-γ (D, G). ****p < 0.0001, by one-way ANOVA test.

Effect of senescence on pulp cells migration and proliferation in immunoinflammatory conditions

It was observed that senescent cells had a lower migratory capacity compared to non-senescent cells in all tested conditions and during both experimental periods. Additionally, a decrease in the proliferative capacity of senescent cells was observed compared to non-senescent pulp cells. Furthermore, the immunoinflammatory stimulus in non-senescent cells led to an increase in proliferation compared to the untreated cells, which was not observed in the senescent cells. This suggests that non-senescent cells retain the ability to respond to the immunoinflammatory stimulus with an increase in proliferation, while senescent cells lose this ability, reflecting a compromised functional state (Figure 3).

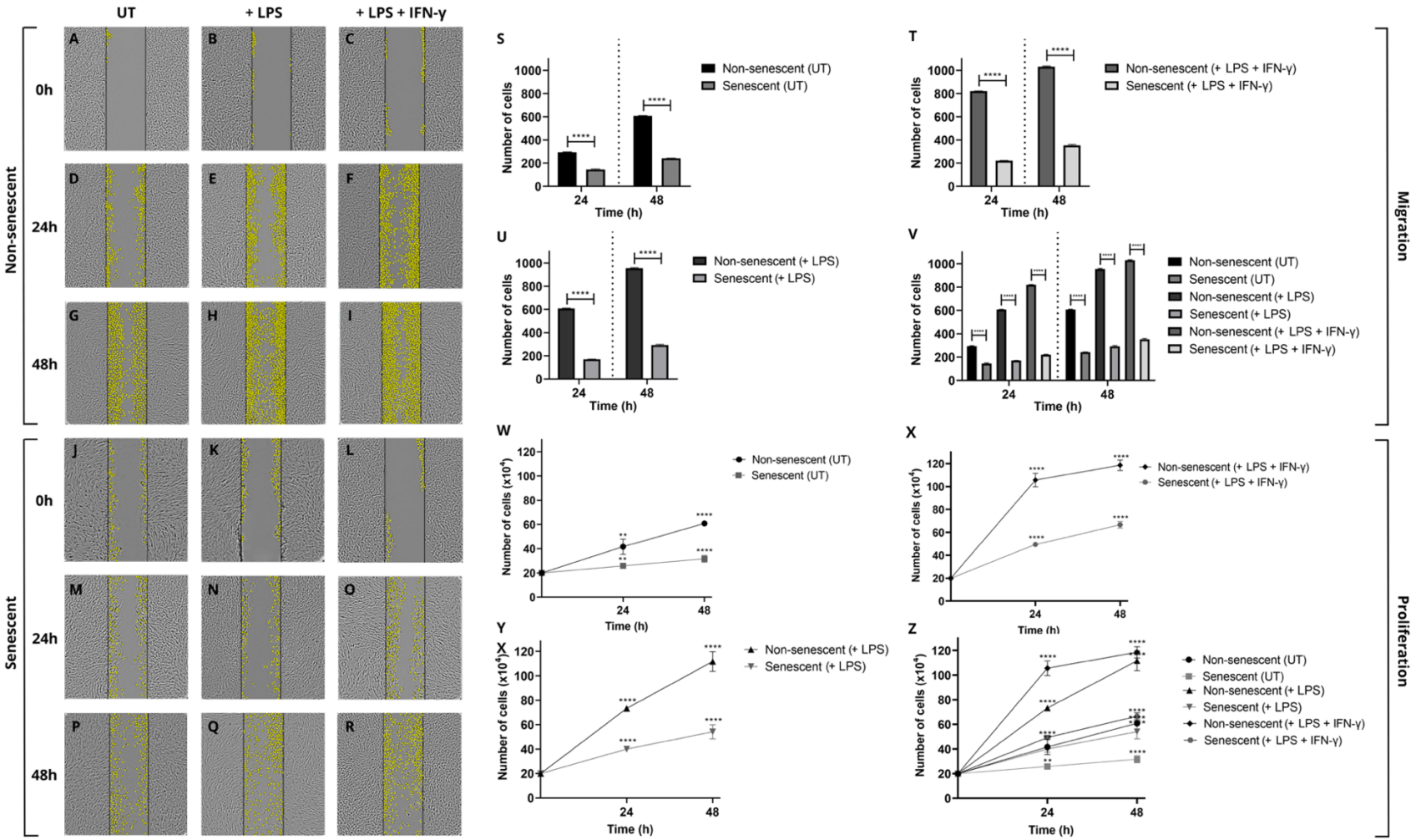

Figure 3. Migration and proliferation of senescent and non-senescent pulp cells under different conditions. Representative images of the migration: Non-senescent: untreated (UT) (A, D, G), in the presence of LPS (B, E, H), stimulated by LPS plus IFN-γ (C, F, I); Senescent: UT (J, M, P), in the presence of LPS (K, N, Q), stimulated by LPS plus IFN-γ (L, O, R). Time points: 0h (A, B, C, J, K, L), 24h (D, E, F, M, N, O), and 48h (G, H, I, P, Q, R). Yellow dots denote the presence of cells within the wound. Graphical representation of the data observed in the migration and proliferation assays: UT (S, W), groups in the presence of LPS (U, Y), groups stimulated by LPS plus IFN-γ (T, X), and comparative analysis between all assay groups (V, Z). **p <0.0013 and ****p <0.0001, by one-way ANOVA test.

Effect of senescence on pulp cells production of inflammatory and anti-inflammatory mediators

To evaluate the inflammatory response, anti-inflammatory response, and repair capacity, the expression of *IDO, TNF-α, IL-6, IL-10,* and *TGF-β1* in senescent pulp cells was measured and compared with non-senescent pulp cells (Figure 4). Increased *IDO* expression was observed in senescent pulp cells compared to non-senescent pulp cells under all tested conditions (UT: *p <0.0245; LPS: *p <0.0245; LPS plus IFN-γ: ****p <0.0001). Moreover, increased *TNF-α* expression was observed in senescent pulp cells compared to non-senescent pulp cells in the LPS plus IFN-γ stimulated group (**p <0.0013), with no statistical differences in other conditions. Similarly, *IL-6* expression was also up-regulated by senescent pulp cells compared to non-senescent pulp cells under all tested conditions (UT: *p <0.0245; LPS: **p <0.0013; LPS plus IFN-γ: ****p <0.0001). Additionally, an increase in *IL-10* expression was observed in senescent pulp cells compared to non-senescent pulp cells; however, no statistical difference was observed in all conditions. In contrast, reduced *TGF-β1* expression was observed in senescent pulp cells compared to non-senescent pulp cells in the LPS-treated group (**p <0.0013), with no statistical differences in other conditions. Senescent pulp cells exhibit a distinct inflammatory profile characterized by increased expression of pro-inflammatory mediators, such as IDO, TNF-α, and IL-6, particularly under immune-inflammatory stimulation. The elevated levels of IL-10, despite the lack of statistical significance in all conditions, suggest a potential compensatory anti-inflammatory response. Additionally, the reduction in TGF-β1 expression under LPS and LPS plus IFN-γ stimulation indicates an impaired reparative capacity in senescent cells. Together, these results highlight the pro-inflammatory phenotype and compromised reparative potential of senescent pulp cells in immune-inflammatory microenvironments (Figure 4).

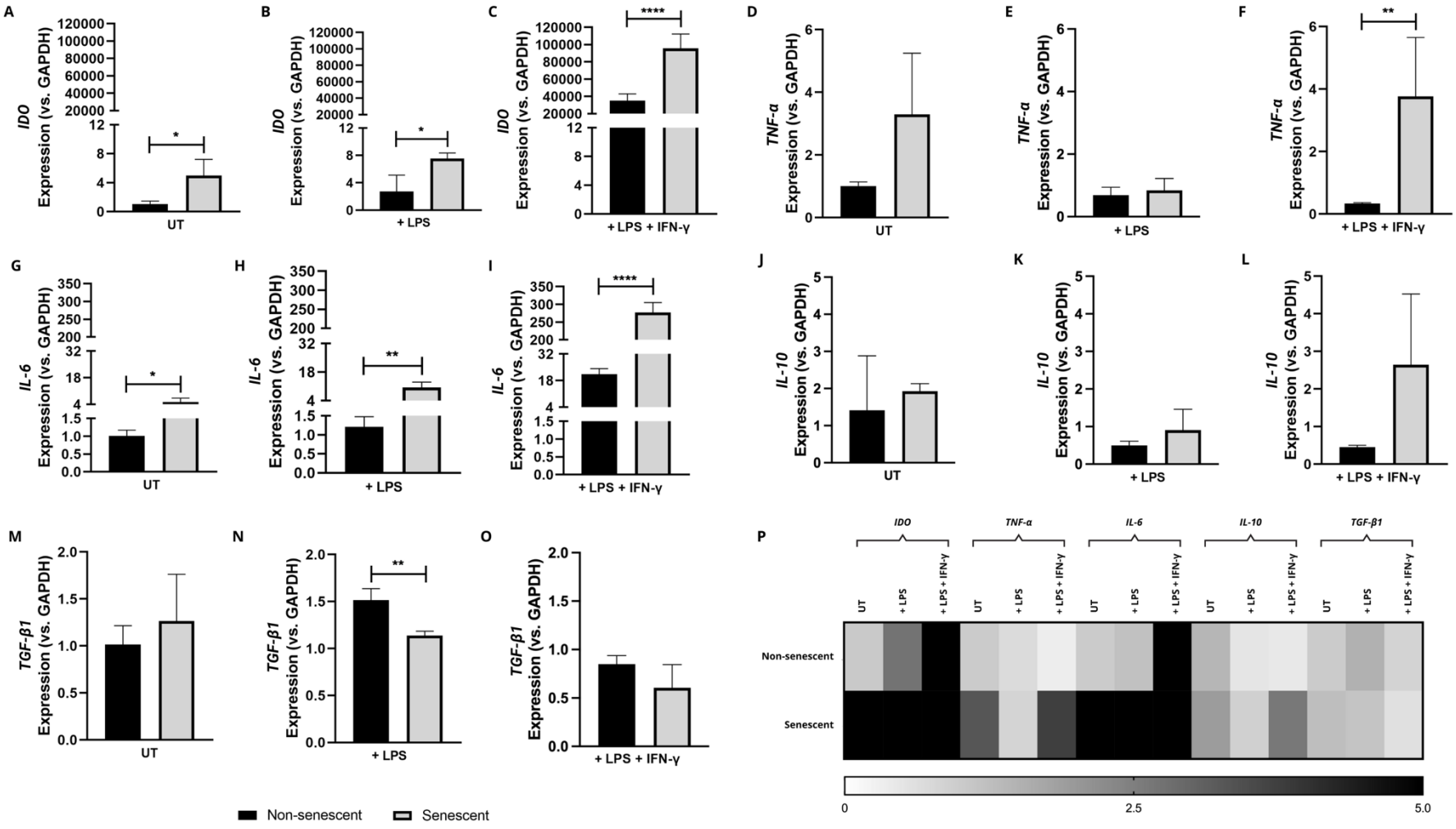

Figure 4. Gene expression of inflammatory and anti-inflammatory mediators by senescent and non-senescent pulp cells in different experimental conditions. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* was used as constitutive gene. *IDO* expression (A, B, C); *TNF-α* expression (D, E, F); *IL-6* expression (G, H, I); *IL-10* expression (J, K, L); and *TGF-β1* expression (M, N, O), by senescent and non-senescent pulp cells. Heatmap indicating the variation in *IDO*, *TNF-α*, *IL-6*, *IL-10*, and *TGF-β1* expression (P). Experimental conditions: Untreated cultures (UT), LPS-stimulated cultures (+LPS), and LPS plus IFN-γ-stimulated cultures (+LPS+IFN-γ). *p <0.0245, **p <0.0013 and ****p <0.0001, by one-way ANOVA test.

DISCUSSION

In the present study, pulp cell senescence was reproduced *in vitro* through stimulation with doxorubicin, a chemotherapeutic agent widely used in the literature to induce this state in various cell types, including fibroblasts and dental pulp cells (20, 10). This method offers advantages such as reproducibility and a well-characterized mechanism of senescence induction through DNA damage and cell cycle arrest. However, it differs from senescence resulting from natural aging, as it is triggered by acute stress rather than a gradual process, resembling senescence induced by trauma, inflammation, pulp infections, and dental caries. Despite this limitation, doxorubicin-induced senescence serves as a valuable model for investigating cellular and tissue responses under conditions that mimic aged or chronically inflamed pulp tissues. Thus, this high percentage of β-galactosidase-stained cells suggests that the adopted protocol was efficient in inducing senescence, ensuring the validity of subsequent assays (21, 6).

Nonetheless, cell viability analysis revealed that senescent pulp cells exhibited a significant reduction in the number of live cells compared to non-senescent pulp cells under all tested conditions. Furthermore, when exposed to immunoinflammatory stimuli (LPS and LPS plus IFN-γ), viability was drastically reduced. Therefore, these findings indicate that senescent pulp cells have a lower resistance to inflammatory challenges, which may be related to cellular metabolism, increased production of reactive oxygen species (ROS), and a senescence-associated secretory phenotype (SASP), characterized by the release of pro-inflammatory cytokines and degenerative factors (22).

Additionally, morphological changes were observed in senescent pulp cells, corroborating previous literature findings that associate this process with significant structural modifications, such as increased, flattened, multinucleated, and vacuolated cells (23). Thus, the parameters considered in the quantitative analysis of the scanning electron microscopy assay included cell size and extensions (6). A marked increase in the size of senescent pulp cells was observed, which may be related to cytoskeletal alterations and intracellular organization, characteristics previously described in senescent cells of different types (24). These changes were evident both under basal

conditions and immunoinflammatory stimuli (LPS and LPS plus IFN-γ), suggesting that senescence impacts cell morphology regardless of the presence of inflammation. Given this, the findings reinforce the idea that the senescent phenotype may be a determining factor in cellular responses to the microenvironment, potentially influencing biological processes such as regeneration and tissue repair (5).

Moreover, a significant reduction in the number of cellular extensions was observed in senescent pulp cells, which may indicate a decreased capacity for cell interaction and communication. Cellular extensions play an essential role in extracellular matrix remodeling and intercellular signaling, being fundamental for processes such as cell migration and inflammatory response (25). Thus, the reduction in these structures may suggest a functional impairment of senescent cells, directly impacting tissue homeostasis and the regenerative capacity of the dental pulp (1). Additionally, it is known that the senescence-associated secretory phenotype (SASP) may contribute to cytoskeletal modifications and cell adhesion, reflecting the altered morphology observed (26). However, the functional implications of these changes are not yet fully elucidated.

In the evaluation of cell migration and proliferation, we observed that senescent pulp cells exhibited lower migratory and proliferative capacity at 24 and 48 hours compared to non-senescent cells, regardless of the experimental condition evaluated. Cell migration is a fundamental process for repair and for maintenance of homeostasis, and the reduction of this capacity in senescent cells may particularly compromise the regenerative response of the dental pulp (27). In this context, previous studies with pulp cells have already demonstrated that senescence is associated with a decline in migratory capacity, which corroborates our findings (28, 6). Another important aspect was that this migration deficit was also observed in cells stimulated by LPS and LPS plus IFN-γ, indicating that the inflammatory process was not sufficient to restore the migratory capacity of senescent cells, in contrast to the results obtained in non-senescent pulp cells.

We also observed a reduction in the proliferation of these cells, which aligns with the literature describing senescence as a state of irreversible cell cycle arrest. Consequently, it is evident that this decline in proliferation may directly impact the ability of dental pulp to respond to injuries, making this tissue less efficient in repair and regeneration (29). Furthermore, the results showed that while non-senescent cells

responded to the immunoinflammatory stimulus with an increase in proliferation, this response was not observed in senescent cells (30, 6). This finding suggests that senescent cells lose the ability to adapt their proliferative activity in response to inflammatory challenges, characterizing the extent of impairment associated with this state.

Regarding the immunoinflammatory response, we evaluated the expression of IDO, TNF-α, IL-6, IL-10, and TGF-β1 in senescent and non-senescent pulp cells under different inflammatory conditions, which could provide us with concrete evidence about the inflammatory phenotype and the compromised reparative potential of senescent cells. Regarding the results, there was a significant increase in IDO expression by senescent pulp cells, when compared to non-senescent cells in all conditions tested. IDO plays a crucial role in the regulation of the immune response through the degradation of tryptophan and can modulate inflammation and promote immunological tolerance (31). Therefore, the results obtained suggest that its increased expression, especially in inflammatory contexts, may reflect a compensatory mechanism to limit tissue damage and prevent exacerbated immune responses (32). However, its increased expression in senescent cells may indicate an ineffective attempt to control chronic inflammation, a characteristic of the inflammaging process.

On the other hand, the increase in TNF-α expression by senescent pulp cells corroborates the results already reported in the literature. TNF-α is a key cytokine in the inflammatory response, playing a central role in the activation of the innate and adaptive immune system, and it is considered a marker of early inflammation and directly related to inflammaging (33, 34). In the study, we observed a significant increase in TNF-α expression in senescent pulp cells, especially in the group stimulated with LPS plus IFN-γ, which contributes to a senescence-associated secretory phenotype (SASP) and the persistence of tissue inflammation. In addition to regulating apoptosis and immune cell activation, TNF-α can induce the expression of other inflammatory cytokines, reinforcing a chronic inflammatory microenvironment (1). Therefore, overexpression of this cytokine may be indicative of impaired tissue repair capacity and immune response, which directly affects the maintenance of dentinopulpal tissue homeostasis and the response to inflammatory stimuli.

IL-6, in turn, was also significantly expressed in senescent pulp cells in all conditions tested, indicating an exacerbated inflammatory response of these cells.

Indeed, IL-6 is a cytokine known for its dual function, acting both as a mediator of inflammation and in the regulation of tissue homeostasis (35). In addition, it is considered one of the main mediators of host response after aggression. Thus, the higher expression of this cytokine in senescent cells compared to the non-senescent group suggests an exacerbated inflammatory profile (36). This result corroborates the “inflammaging” scenarios, in which the expression of cytokine IL-6 is also increased. Furthermore, our findings indicate a greater susceptibility of these cells to persistent inflammatory processes, favoring a microenvironment less prone to inflammatory resolution and more predisposed to chronic inflammatory complications (6).

Furthermore, although a tendency towards increased expression of IL-10 in senescent cells was observed, this difference was not statistically significant in any of the conditions tested. IL-10 is an anti-inflammatory cytokine essential for regulating the immune response and controlling excessive inflammation (37, 38). The absence of a significant increase in the expression of this cytokine may indicate a limited capacity of senescent cells to efficiently modulate the inflammatory response, which may compromise the immune balance and boost tissue damage (39). This fact may suggest a slower reestablishment of the inflammatory response balance, which could possibly affect the prognosis in a context of conservative treatment.

Finally, the expression of TGF-β1 was reduced in senescent cells in the presence of inflammatory stimuli, especially in the group stimulated with LPS, suggesting a decrease in the capacity for regenerative response. TGF-β1 is a decisive cytokine in cell proliferation, differentiation and regulation of the immune response, playing an important role in inflammatory resolution and tissue repair (40). Studies indicate that reduced TGF-β1 expression may be associated with a senescent phenotype that is less responsive to regenerative signals, which could negatively impact the pulp repair capacity in the face of injuries and inflammatory or infectious stimuli (6, 41). This factor may also negatively impact the success of conservative endodontic treatments in senescent pulps.

In summary, the results of this study demonstrate that cellular senescence is a limiting factor in the regenerative and reparative capacities of dental pulp, especially in scenarios of inflammation and infection. Senescent pulp cells showed impairment of essential aspects such as viability, migration, proliferation and immunoinflammatory response, in addition to undergoing morphological changes that suggest a reduced

function of the pulp in regenerative processes. The modulation of inflammatory pathways and the decrease in the expression of regenerative cytokines, such as TGF-β1, indicate a decreased tissue repair capacity, which may directly impact the success of conservative endodontic treatments. These findings highlight the importance of considering the state of cellular senescence in therapeutic strategies, particularly in patients with compromised pulps, where the implementation of approaches aimed at reversing or mitigating senescence may be crucial to improving clinical outcomes.

Future studies should investigate the potential of new senotherapeutic molecules for dentistry, as well as the feasibility of interventions that promote the prevention or treatment of cellular senescence, aiming for better outcomes in conservative and regenerative endodontic treatments. Furthermore, in the clinical context, it is essential to adapt treatment protocols, prioritizing techniques that minimize pulp stress and promote the maintenance of dental pulp homeostasis, in order to improve the response to these treatments.

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CAPÍTULO 3 (Intended submission: Peptides; IF = 2.8)

Senotherapeutic peptides as a promising strategy for modulating cellular senescence in dental pulp cells

ABSTRACT

Cellular senescence plays a crucial role in the decline of pulp tissue functions, compromising the success of conservative endodontic treatments and directly impacting the homeostasis of the dentin-pulp complex. In this study, an initial three-step screening of a library comprising 15 peptides was performed to identify senotherapeutic molecules using the β-galactosidase staining assay, as well as to evaluate cytotoxicity and nitrite production in dental pulp cells before and after senescence induction with doxorubicin. Peptides C3 and C10 demonstrated a significant reduction in cellular senescence, with C3 showing the highest inhibition rate (61%) prior to senescence induction. Both peptides exhibited good biocompatibility and safety at tested concentrations. Furthermore, a positive impact on nitrite production modulation was observed, suggesting a potential effect on the inflammatory microenvironment. When compared to other senotherapeutic peptides reported in the literature, C3 and C10 displayed similar efficacy, with the added advantage of acting both preventively and therapeutically. Thus, peptides C3 and C10 emerge as promising candidates for the development of senotherapeutic therapies targeting cellular senescence in pulp tissue. However, additional studies are required to refine their properties and gain a better understanding of the mechanisms underlying their actions.

Keywords: Dental Pulp Cells; Cellular Senescence; Senotherapeutics; Peptides.

INTRODUCTION

Senescent cells accumulate in numerous tissues of the human body with aging and contribute to age-related pathologies. This accumulation is also potentiated by stress factors, which can accelerate the process of cellular senescence (Jeon et al., 2017). Senescent cells secrete a variety of factors that promote the development of the senescence-associated secretory phenotype (SASP), contributing to chronic inflammation and the worsening of various pathological conditions (Zonari et al., 2023). Aging of dental pulp tissue is a complex process influenced by a variety of intrinsic and extrinsic factors. Intrinsically, chronological age plays a significant role, while extrinsic factors such as dental caries, bruxism, trauma, periodontal disease, and the use of certain dental products further contribute to the aging process. These factors collectively impact cellular function and tissue regeneration within the dental pulp, potentially leading to diminished pulp vitality and altered tissue responses (Farias et al., 2024).

The presence and accumulation of senescent cells in the pulp tissue have been widely discussed in the literature and have serious implications for dentistry (Farias et al., 2023). In endodontics, conservative and regenerative treatments have shown promise for preserving dental function and recovering pulp tissue. While conventional treatments focus on eliminating infection and restoring the integrity of the tooth through disinfection and obturation of the root canal system, conservative treatments aim to promote the maintenance and regeneration of pulp tissue (Yong et al., 2021). However, it is essential to balance the defense responses of the pulp tissue so that regeneration and repair of the dentin-pulp complex occur effectively (Yu et al., 2007).

Thus, senescence of pulp tissue can compromise its various functions, including formative, nutritive, defensive, and sensory capacities (Galler et al., 2021). This condition can directly affect the success of conservative endodontic treatments, highlighting the importance of developing senotherapeutic approaches focused on preserving the health of the dentin-pulp complex (Farias et al., 2024). Despite advances in understanding cellular senescence and SASP

modulation, this knowledge has not yet been fully translated into effective interventions for treating or preventing this condition (Zonari et al., 2023). This gap represents a significant challenge, especially in the context of dentistry, where the search for strategies to prevent or treat this state is crucial to improving clinical outcomes.

So far, no study has described the effects of senotherapeutic molecules designed to modulate the senescence process in the dentin-pulp complex. However, several senolytics and senomorphics have already been reported in the literature for their ability to mitigate senescence in other tissues (Di Micco et al., 2021). Among these molecules, the FOXO4-DRI peptide stands out, an antagonist designed to interrupt the interaction between FOXO4 and p53, promoting the nuclear exclusion of p53 and its relocation to mitochondria, where it induces apoptosis in senescent cells (Baar et al., 2017). Moreover, dasatinib and quercetin have shown effectiveness in reducing cellular senescence levels in the skin of patients with diabetic kidney disease (Hickson et al., 2019). The OS-01 peptide (or peptide 14) has been shown to reduce the senescence burden in three-dimensional human skin models and *ex vivo* skin samples, promoting health markers and significantly reducing the biological skin age (Zonari et al., 2024). Additionally, the UBX0101 peptide, an inhibitor of the interaction between p53 and MDM2, has been identified as a potential inducer of apoptosis in senescent cells (Lane et al., 2021).

The evolution of rational peptide design has been greatly driven by technological advancements that allow the creation of highly specific and effective bioactive molecules (Kaynak, 2021). Initially, peptide development was guided by time-consuming experimental processes based on trial and error (Mata et al., 2023). However, with the advent of advanced computational tools, the approach has become more targeted, leveraging structural modeling and molecular simulations to predict interactions between peptides and their biological targets (Hashemi et al., 2024). The emergence of artificial intelligence (AI) has taken this field to a new level, enabling not only the prediction of peptide sequences with desired activities but also the optimization of critical parameters such as stability in biological media, bioavailability, resistance to enzymatic degradation, and selectivity for specific targets (Kaynak, 2021). AI-based tools,

such as machine learning algorithms and deep neural networks, have been widely used to analyze large volumes of biological data, identifying complex patterns that would be difficult to detect using conventional methods (Lavecchia, 2019).

The application of artificial intelligence (AI) in the development of senotherapeutics encounters several significant challenges, particularly the limited availability of high-quality datasets pertaining to peptide sequences for the training of predictive models (Li et al., 2022). Nevertheless, numerous antimicrobial peptides exhibit the capacity to interact with multiple cellular targets and modulate a range of biological pathways. This multifaceted activity represents a notable advantage, and such peptides are often referred to as promiscuous peptides due to their broad-spectrum functionality. This characteristic makes them particularly promising in different fields, as they can exert varied actions, such as anti-inflammatory, immunomodulatory, and even anti-senescent activities (Amorim et al., 2021; Lima et al., 2017). Furthermore, these peptides can act on crucial processes, such as inflammation, oxidative stress, and DNA repair, which are closely linked to cellular senescence (Kumar et al., 2018).

Therefore, this study aimed to conduct an initial screening of a library of antimicrobial peptides in search of molecules with senotherapeutic potential. Based on the results obtained, the therapeutic potential of the new C3 and C10 peptides in combating cellular senescence was highlighted, offering a promising approach for treating tissues compromised by senescence, such as pulp tissue. The findings that C3 and C10 can mitigate senescence in pulp cells support the idea that promiscuous antimicrobial peptides can perform different biological functions beyond their initial target. Finally, although the results are promising, it is essential to conduct further studies to optimize the specificity, potency, and stability of these peptides using artificial intelligence tools.

MATERIAL AND METHODS

In silico design of the peptides

The sequence of chosen peptides was designed by artificial intelligence. For this, available peptide sequence databases were used to train and test predictive models. A positive dataset, with 684 sequences, was extracted from GRAMPA (Giant Repository of AMP Activities) and filtered using CD-hit to eliminate redundancies. Additionally, a negative dataset was obtained from UniProt database, excluding non-standard residues, also filtered by CD-hit and randomly reduced to 684 sequences to form a final dataset. Both datasets were unified, totaling 1,368 sequences for model training. Then, a scoring function was developed for predicting antimicrobial activity, and a convolutional neural network (CNN) was implemented as an encoder, with a multi-layer perceptron (MLP) layer for binary classification. The created database was randomly divided into 80% for training and 20% for evaluation and used for model training and evaluation. After that, the conformational space of antimicrobial activity was defined, limiting the sequence length to 16 amino acids, restricting aspartic acid to zero and lysine to at least three, in order to obtain cationic peptides with higher activity. The process is interactive, adjusting restrictions to generate effective variants. The agent used to generate new sequences was an LSTM-LM (Long Short-Term Memory-Language Models) model, trained on the SwissProt database and adjusted to generate sequences with antimicrobial activity using the positive database. The PPO gradient method (Proximal Policy Optimization) was employed, and the stability of the sequences generated was assessed with the ESM-2 model. The reward assigned to the sequences, based on classification and stability, ranges from -10 to 10. The AMP-BERT model, based on the Hugging Face Transformers API and ProtBERT-BFD, was used to validate the classification of the newly generated sequences. After fine-tuning with the previously created database, the model was trained and evaluated (80% and 20%, respectively) to predict antimicrobial activity. Sequences with probabilities ≥ 0.5 were classified as antimicrobial, and those with lower probabilities, as non-antimicrobial. Sequences with rewards > 9.5 in the MRL model and predictions ≥ 0.8 by AMP-BERT were selected. ToxinPred 3.0 and HemoPred discarded toxic or hemolytic sequences.

Only sequences with a net charge ≥ +2 were retained (Fernandes el al., 2023). Using this methodology, 15 peptides were selected (sequences not listed due to intellectual property protection) (Table 2).

Peptide	Molecular Formula	Mass Spectrum
C1	$C_{65}H_{119}N_{19}O_{17}$	1438,71
C2	$C_{63}H_{114}N_{18}O_{14}$	1347,61
C3	$C_{69}H_{127}N_{21}O_{14}$	1474,82
C4	$C_{67}H_{128}N_{22}O_{14}$	1466,04
C5	$C_{65}H_{117}N_{21}O_{16}$	1449,17
C6	$C_{65}H_{120}N_{20}O_{17}S$	1486,41
C7	$C_{61}H_{109}N_{19}O_{15}$	1349.80
C8	$C_{63}H_{117}N_{19}O_{19}$	1445,83
C9	$C_{63}H_{116}N_{22}O_{16}S$	1469,49
C10	$C_{66}H_{125}N_{19}O_{16}S$	1472,71
C11	$C_{66}H_{125}N_{19}O_{17}S$	1488,74
C12	$C_{63}H_{119}N_{19}O_{17}S_2$	1476,84
C13	$C_{66}H_{125}N_{21}O_{15}$	1453,22
C14	$C_{64}H_{119}N_{21}O_{14}$	1407,37
C15	$C_{59}H_{109}N_{19}O_{14}$	1309,47

Table 1. A library of 15 peptides with antimicrobial potential, including the peptide name, molecular formula and mass spectrum.

Peptide synthesis and determination of molecular mass by MALDI-ToF mass spectrometry

The 15 selected peptides were acquired from SYNBIOTech (New York, USA) with a purity greater than 95%. The purified peptides were qualitatively analyzed using a MALDI-ToF UltraFlex III mass spectrometer (Bruker Daltonics). For the analyses, the lyophilized peptides were dissolved in ultrapure water, mixed with a saturated solution of a matrix composed of α-cyano-4-hydroxycinnamic acid (1:3), deposited onto an Anchorchip plate, and allowed to crystallize at room temperature. Calibration was performed using Peptide

Calibration Standard II (Bruker Daltonics) as molecular mass standards, with data acquired in positive reflective mode (RP_700-3000 Da) (Supplementary Figures 1-4).

Primary Culture of Dental Pulp Cells

Pulp tissues were obtained from intact third molars extracted from donors aged between 18 and 30 years, after approval by the University of Brasília ethics committee (CAAE: 75393923.1.0000.0030). In a laminar flow hood, teeth were washed with phosphate-buffered saline (PBS) and fractured using orthodontic pliers (Quinelato, Rio Claro, São Paulo, Brazil). Coronal and radicular dental pulp was carefully removed using manual K-type endodontic files (Dentsply Sirona, Charlotte, North Carolina, USA). Dental pulps were washed with PBS containing 100 $U.mL^{-1}$ of penicillin (Invitrogen, Grand Island, New York, USA), 100 $\mu g.mL^{-1}$ of streptomycin (Invitrogen), and 1% amphotericin B (Invitrogen). Then, pulp tissue was minced with a scalpel blade and fixed in 6-well cell culture plates (Kasvi, São José dos Pinhais, Paraná, Brazil). Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, San Luis, Missouri, USA) supplemented with 20% fetal bovine serum (FBS) (GIBCO, Grand Island, New York, USA), 100 $\mu g.mL^{-1}$ penicillin (Invitrogen), 100 $\mu g.mL^{-1}$ streptomycin (Invitrogen), and 2 $\mu g.mL^{-1}$ glutamine (GIBCO) was added to each well. Plates were maintained in a humidified atmosphere containing 5% CO_2 at a temperature of 37 °C. Cells were subcultured as they reached confluence to obtain enough cells for each experiment (Naz et al., 2019).

Screening of a peptide library

Since several peptides have more than one activity, often being called promiscuous peptides, the senotherapeutic potential of these peptides was evaluated. For this purpose, an initial screening of the library of 15 peptides obtained was performed, analyzing the presence of senotherapeutic molecules by measuring the beta-galactosidase staining associated with senescence (SA-BGal) in human pulp cells before and after induction of senescence with

doxorubicin at 500 μM (Doxorubicin hydrochloride, Sigma Aldrich) (Figure 1). Pulp cells were seeded in 96-well plates (Kasvi) at a density of $2x10^4$ cells/well. The peptide library was added at final concentrations of 50 μM, 12.5 μM, 5 μM, and 500 nM in DMEM (Sigma Aldrich) without FBS. After 30 minutes, 10% of FBS (GIBCO) was added to the culture. Then, cells were incubated for 48 hours at 37 °C and 5% CO_2. Senescent cells were used as a positive control, and non-senescent cells were considered negative control, both without peptide-treatment. In experimental groups, after peptides-treatment, cells were washed twice for 30 seconds with PBS. Immediately after, cells were fixed with 2% formaldehyde (Dinâmica, Indaiatuba, São Paulo, Brazil) and 0.5% glutaraldehyde (Dinâmica), for 5 minutes at room temperature. Subsequently, 1 mL of the X-Gal staining solution [40 mM citric acid solution (Dinâmica) and sodium phosphate (Sigma Aldrich), 5 mM potassium hexacyanoferrate (Sigma Aldrich), 150 mM sodium chloride (Sigma Aldrich), 2 mM magnesium chloride (Sigma Aldrich), 1 $mg.mL^{-1}$ X-Gal (Thermo Fisher Scientific, Waltham, Massachusetts, USA)] was added, and the plate was incubated at 37 °C, protected from light, overnight. After 16 hours, cells were washed twice for 30 seconds with PBS and once with methanol (Dinâmica). Then, cells were observed and photographed in the central region of each well, always at the same magnification (10X), using an inverted microscope with phase contrast Axio Observer D1 (Zeiss, Oberkochen, Germany). The relative level of senescence was quantified using the CellProfiler software (Cimini Lab, Cambridge, Massachusetts, USA) (Zonari et al., 2023).

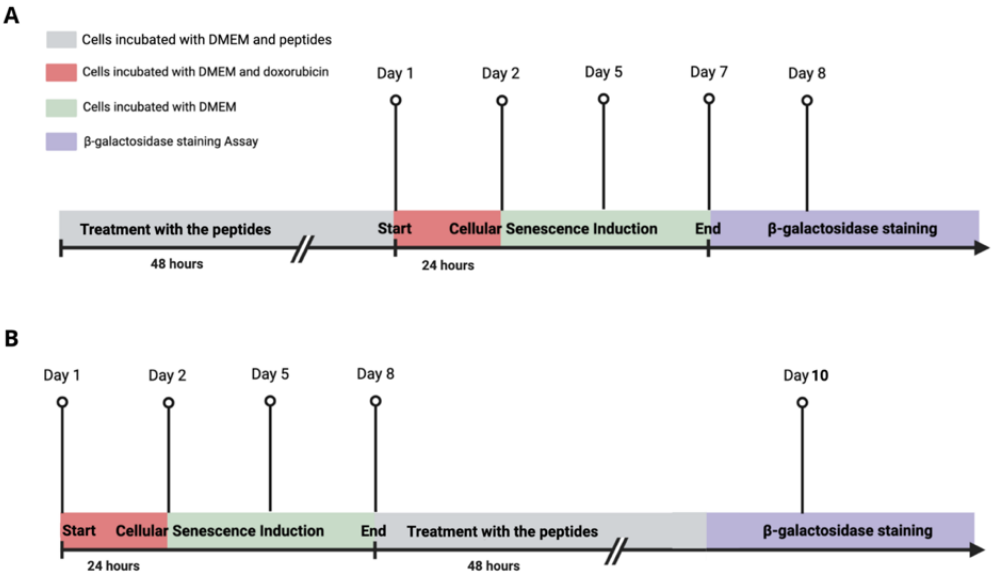

Figure 1. Diagram of the induced senescence protocol and its confirmation by β-galactosidase staining assay, along with peptide treatment. (A) Peptide treatment prior senescence induction. (B) Peptide treatment after senescence induction.

Cell viability

To assess cell viability after 48 hours of incubation with the peptides, the colorimetric MTT assay (Sigma-Aldrich) was used. This method is based on evaluating the activity of the mitochondrial dehydrogenase enzyme. After the incubation period, the supernatant was removed, and 100 µL of DMEM medium (Sigma-Aldrich) was added per well. Then, 10 µL of MTT (5 mg·mL^{-1}) was added to each well. Plates were incubated for 4 hours in 5% CO_2 at 37 °C and 95% humidity. After this period, the reaction was blocked by adding 60 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) per well and homogenized to ensure complete solubilization of the cellular contents. Absorbance was then measured at 570 nm using an Elisa reader (Bio-Tek PowerWave HT, USA). Cell viability in the experimental groups was compared to the positive control group, represented by cell culture in medium (considered 100% viability). Negative control was represented by cell culture in lysis solution (10 mM Tris pH 7.4, 1 mM EDTA, and 0.1% Triton X-100) (Mosmann et al., 1983).

Nitric oxide production

After 48-hour of cell incubation, culture supernatant obtained from both groups, before and after the induction of senescence with doxorubicin (Sigma Aldrich), was collected and evaluated for the presence of NO. Nitrite production in the culture supernatants was assessed using the Griess reaction, and it was assumed to reflect the NO levels. Briefly, 50 µL of culture supernatants were placed in 96-well plates, followed by the addition of 50 µL of a mixture containing 1% sulfanilamide (Sigma-Aldrich) in 2.5% phosphoric acid (Vetec, Brazil) and 1% N-(1-naphthyl)ethylenediamine (Sigma-Aldrich) in 2.5% phosphoric acid (Vetec), at a 1:1 ratio. After 10 minutes of incubation at room temperature, the reading was performed on the ELISA reader (Bio-Tek PowerWave HT) at 490 nm. The nitrite amount was calculated using a sodium nitrite standard curve (200 µM–1.5625 µM) (Green et al., 1982).

Statistical Analysis

All experiments were performed in triplicates. Data were described as means and standard deviations. According to the statistical analysis, normal distribution was verified, and statistical analysis was conducted. All experiments were analyzed using one-way ANOVA. All analyses were performed using GraphPad Prism 10 software and considered at a 95% significance level.

RESULTS

Senotherapeutic Peptides for Prevention and Modulation of Cellular Senescence

A library consisting of 15 peptides, designed using artificial intelligence, was synthesized and screened for the presence of senotherapeutic molecules, utilizing senescence-associated β-galactosidase (SA-βGal) staining in pulp cells before and after senescence induction by doxorubicin. Peptides with the highest senotherapeutic potential and lowest cytotoxicity in this initial screening should

be validated in additional experiments in future studies. In this context, we considered peptides with up to 50% senescence inhibition as promising candidates. The treatment with peptides prior senescence induction was encouraging, being the peptide C3 (500 nM) the one which presented the highest inhibition rate, reaching 61%. This performance was followed by peptides C10, C1, C4, and C12, which inhibited senescence by 58%, 57%, 56%, and 51%, respectively. These findings suggest that these peptides have protective properties, capable of mitigating the effects of doxorubicin on the senescence process (Figure 2). However, during the analysis of peptide treatment after senescence induction, only peptides C3 (12.5 μM) and C10 (500 nM) demonstrated an effect on reducing senescence levels, with inhibition rates of 48% and 49%, respectively. This finding is particularly relevant as it suggests that these peptides not only prevent senescence but may also modulate cellular processes associated with established senescence (Figure 3). The overall analysis indicates that peptides C3 and C10 stood out as the most promising candidates due to their efficacy both before and after senescence induction, as well as their significant activity even at relatively low concentrations.

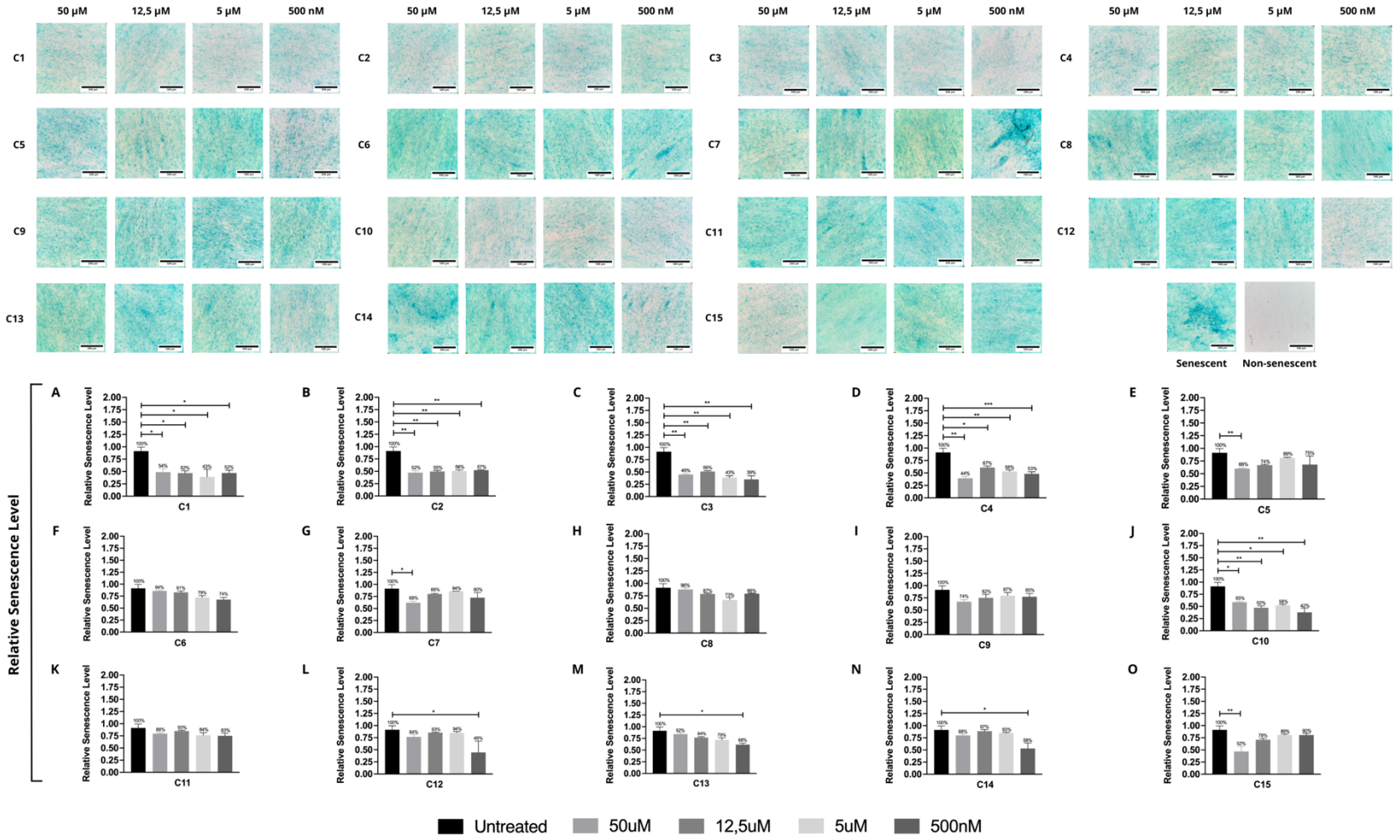

Figure 2. Screening of a library of 15 peptides at different concentrations using the β-galactosidase staining assay, with peptide treatment prior to senescence induction by doxorubicin. Bars represent the following groups: Untreated, 50 µM, 12.5 µM, 5 µM, and 500 nM, respectively. Percentage above each bar indicates the proportion of β-galactosidase-positive cells in each condition, in relation to the untreated group. Peptides: (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, (F) C6, (G) C7, (H) C8, (I) C9, (J) C10, (K) C11, (L) C12, (M) C13, (N) C14, (O) C15. *p < 0.05, **p < 0.005, ***p < 0.0005, by one-way ANOVA test.

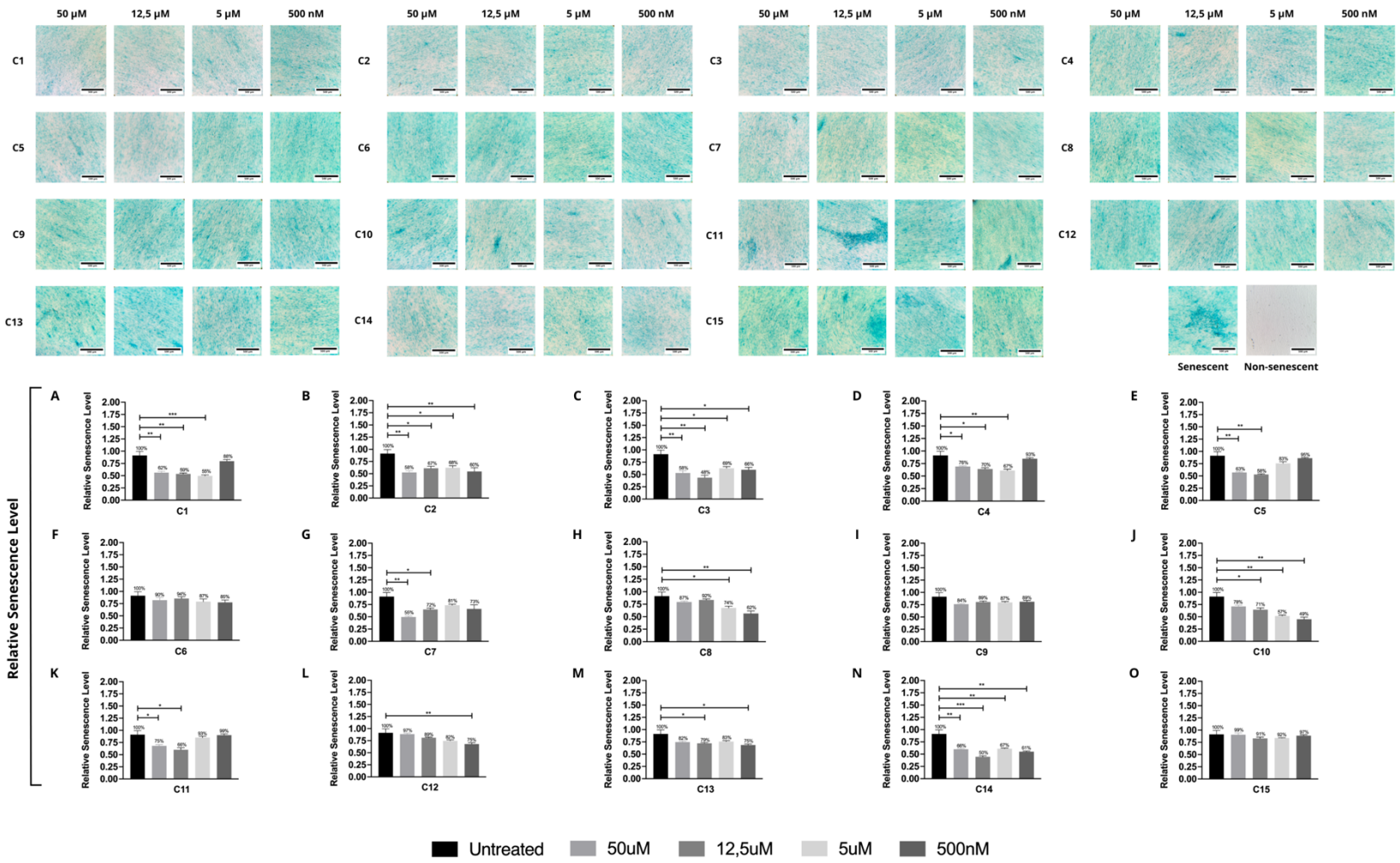

Figure 3. Screening of a library of 15 peptides at different concentrations using the β-galactosidase staining assay, with peptide treatment after senescence induction by doxorubicin. Bars represent the following groups: Untreated, 50 µM, 12.5 µM, 5 µM, and 500 nM, respectively. Percentage above each bar indicates the proportion of β-galactosidase-positive cells in each condition, in relation to the untreated group. Peptides: (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, (F) C6, (G) C7, (H) C8, (I) C9, (J) C10, (K) C11, (L) C12, (M) C13, (N) C14, (O) C15. *p < 0.05, **p < 0.005, ***p < 0.0005, by one-way ANOVA test.

In Vitro Toxicity of Peptides

Human pulp cells were cultured for 48 hours in the presence of different doses of the 15 tested peptides (50 μM, 12.5 μM, 5 μM, and 500 nM) in two distinct experimental groups: treatment with peptides prior doxorubicin-induced senescence and treatment with peptides after doxorubicin-induced senescence. After cell incubation period, cell viability was determined using the MTT assay. It was shown that C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, and C12 peptides were not toxic to pulp cells at any of tested concentrations, when used previously to induced senescence. On the other hand, peptides C11, C13, C14, and C15 showed considerable toxicity at higher concentrations, reducing cell viability by more than 50%, particularly peptide C13 at a concentration of 50 μM. Regarding, peptide toxicity after senescence induction, it was observed a good cell viability in the presence of C2, C3, C4, C8, C9, C10, C13, C14, and C15 peptides, while C1, C5, C6, C7, C11, and C12 peptides exhibited toxicity, especially at higher concentrations (Figure 4). These data suggest that peptides C3 and C10, identified as potentially senotherapeutic, are not toxic to dental pulp cells, showing that even at higher concentrations, they maintain cell viability, indicating their safety and therapeutic potential.

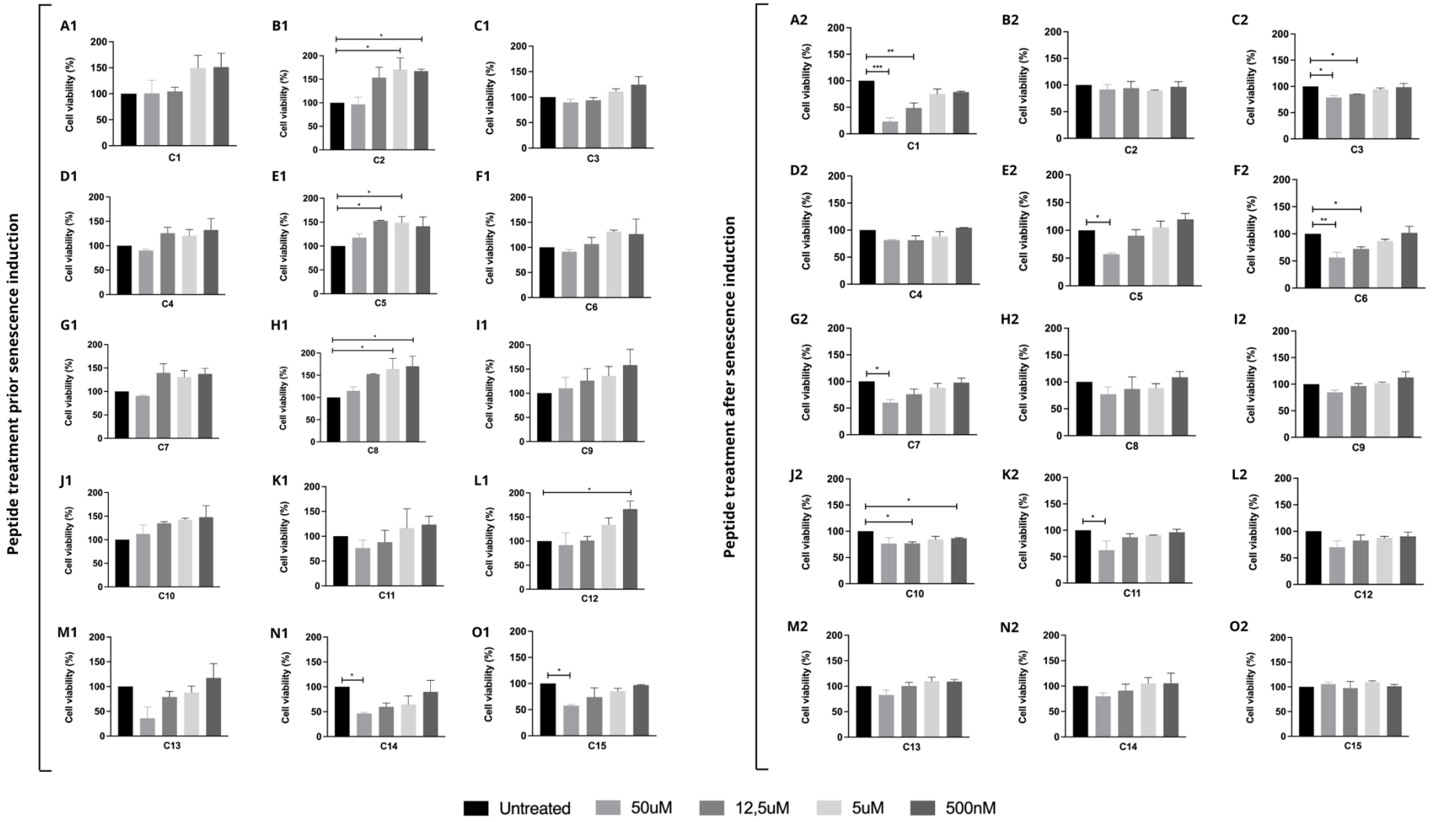

Figure 4. Cell viability of pulp cells treated with a library of 15 peptides at different concentrations, before (1) and after (2) senescence induction. Cell viability of the peptide-treated groups under both conditions was as follows: (A1 and A2) C1, (B1 and B2) C2, (C1 and C2) C3, (D1 and D2) C4, (E1 and E2) C5, (F1 and F2) C6, (G1 and G2) C7, (H1 and H2) C8, (I1 and I2) C9, (J1 and J2) C10, (K1 and K2) C11, (L1 and L2) C12, (M1 and M2) C13, (N1 and N2) C14, (O1 and O2) and C15. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA test.

Nitric oxide production

Nitric oxide production was evaluated in human dental pulp cells treated with a library of 15 peptides at different concentrations, both before and after doxorubicin-induced senescence. Nitrite levels, an indirect marker of nitric oxide, were measured using the Griess assay after 48 hours of incubation. Peptide treatment before senescence induction led to a significant increase in nitrite production compared to the untreated group. In addition, peptide effects on NO production were dose-dependent, being 50 μM the greatest effects compared to 500 nM. In contrast, nitrite levels remained stable without significant variation with increasing doses, staying close to control levels, when peptides treatment happens after cellular senescence induction (Figure 5). None of the 15 peptides resulted in a reduction in nitrite levels relative to untreated group. Notably, peptides C3 and C10, selected in previous analyses, demonstrated the ability to regulate nitric oxide production in senescent cells.

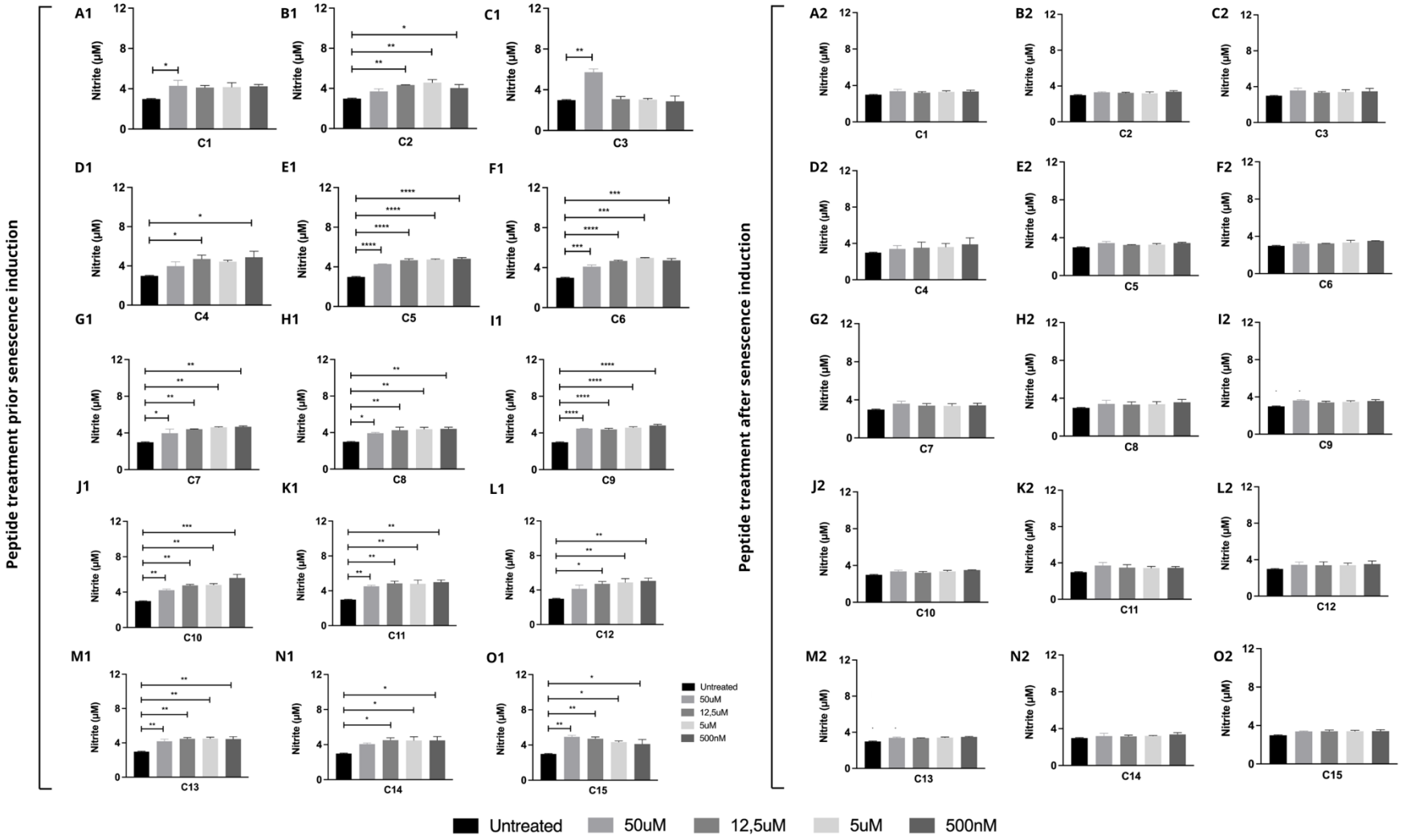

Figure 5. Nitric oxide production of pulp cells treated with a library of 15 peptides at different concentrations, before (1) and after (2) senescence induction. Nitrite levels in the peptide-treated groups under both conditions were as follows: (A1 and A2) C1, (B1 and B2) C2, (C1 and C2) C3, (D1 and D2) C4, (E1 and E2) C5, (F1 and F2) C6, (G1 and G2) C7, (H1 and H2) C8, (I1 and I2) C9, (J1 and J2) C10, (K1 and K2) C11, (L1 and L2) C12, (M1 and M2) C13, (N1 and N2) C14, (O1 and O2) and C15. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ by one-way ANOVA test.

DISCUSSION

The maintenance of pulp tissue health is essential for preserving the functions of the dentin-pulp complex, including its formative, nutritive, defensive, and sensory capacities (Yu et al., 2007). Throughout life, various factors such as caries, bruxism, trauma, periodontal disease, and the use of dental products can cause changes to this tissue (Yu et al., 2007; Jontell et al., 1998), eventually leading to its senescence. Cellular senescence is characterized by an irreversible disruption of the cell cycle, accompanied by morphological, metabolic, lysosomal, and secreted factor changes (González-Gualda et al., 2021). As a consequence, senescent pulp exhibits dysfunction in its defensive and sensory functions, compromising the success of both conservative and regenerative endodontic treatments (Yong et al., 2021).

The search for therapies aimed at reducing cellular senescence has gained prominence, as this condition compromises various vital functions in the body. However, dental research in terms of the development of senotherapeutic products is in its infancy. Although the number of senotherapeutic compounds, both natural and synthetic, is still limited, their use to modulate cellular senescence has shown great potential, especially in tissues susceptible to recurring damage, such as pulp tissue (Zonari et al., 2022). The implementation of therapies capable of maintaining pulp function while reducing the effects of senescence could represent a crucial advancement for endodontics and other areas of dentistry.

Antimicrobial peptides frequently interact with cellular structures due to their physicochemical properties, such as positive charge and amphipathicity, which facilitate interaction with lipid membranes and intracellular components (Kumar et al., 2018). This ability to interact with membranes and cellular pathways can influence other processes as oxidative stress, inflammation, and DNA repair, all of which are strongly involved in cellular senescence (Kumar et al., 2018). Thus, many peptides are considered promiscuous once they have multiple targets and may act on biological pathways beyond their initial function, fact observed in many studies on bioactive peptides (Amorim et al., 2021; Lima et al., 2017).

The rational design of peptides and their subsequent synthesis have become routine practices in the development of promising bioactive molecules. With the advent

of artificial intelligence, this process has expanded even further, allowing the exploration of peptides with diverse and complex biological activities (Kaynak, 2021; Hashemi el al., 2024). By utilizing AI tools, researchers can predict and optimize sequences with specific properties, facilitating the discovery of molecules that interact with multiple cellular pathways. This advancement not only accelerates the identification process of new therapeutic peptides but also enables the adaptation of molecules to interact more effectively with specific cellular targets (Hashemi el al., 2024).

Although machine learning methods generally perform well in predicting new peptides, the lack of detailed peptide sequence data available for training can limit their performance (Li et al., 2022). Therefore, the decision was made to evaluate the presence of senotherapeutic molecules through the design of new antimicrobial peptides, which have greater availability of sequence data. However, the size of the training data remains small compared to the data typically used in deep learning applications, which may have influenced the obtained results.

Thus, these new peptides may be promising options, offering effective and rapid therapeutic action or even lower costs. Currently, there are several antimicrobial peptides whose structure and mechanism of action are well described. However, recent research has focused on developing peptides that, in addition to being antimicrobial, exhibit other functions, such as immunomodulatory, anti-inflammatory, and even senotherapeutic effects. These peptides are called promiscuous peptides, where multiple targets are associated with a single peptide structure (Mulder et al., 2013).

Therefore, this study aimed to evaluate these peptides, focusing on identifying senotherapeutic molecules with potential to mitigate cellular senescence in pulp tissue. The identification and evaluation of a library of 15 peptides designed by artificial intelligence highlight the potential of using advanced computational tools to develop therapies targeting cellular dysfunction related to aging. Notably, peptides C3 and C10 stood out as the most promising candidates due to their dual ability to inhibit senescence before its induction and modulate already established senescence, suggesting a versatile therapeutic profile.

The results revealed significant variability to inhibit senescence, with C3 achieving the highest inhibition rate of 61% when used before senescence induction, followed by peptides C10, C1, C4, and C12. These findings highlight the protective potential of these peptides against senescence triggered by doxorubicin, a well-established chemotherapeutic agent known to induce senescence (Yaghoobi et al., 2020). The superior efficacy of C3 and C10 may be attributed to their specific interactions with molecular targets involved in the senescence pathway, although the precise mechanisms still require further elucidation.

When comparing these results with senotherapeutic peptides described in the literature, such as Peptide 14, which showed approximately 30% inhibition in primary human dermal fibroblasts (HDF) (Zonari et al., 2023), FOXO4-DRI, with over 40% inhibition in human chondrocytes (Li et al., 2022), and UBX0101, with 50% inhibition also in human chondrocytes (Jeon et al., 2017), it is observed that peptides C3 and C10 exhibit similar efficacy in human dental pulp cells. However, direct comparison with other classes of senotherapeutic peptides still requires further studies, particularly regarding cellular specificity and the mechanisms involved in each cell type.

When applied after senescence induction, peptides C3 and C10 maintained their therapeutic potential, reducing senescence levels by approximately 48-49%. This dual functionality is particularly notable as it suggests that these peptides not only prevent the onset of senescence but also possess reparative properties, potentially reversing or attenuating the senescent phenotype. The capacity of these peptides to exert both preventive and modulatory effects highlights their clinical significance in conditions where cellular aging is a contributing factor (Zonari et al., 2023).

In addition to peptides C3 and C10, other senotherapeutic peptides have demonstrated the ability to act both preventively and therapeutically after senescence induction. One example is peptide 14, which, when administered, significantly increased the number of non-senescent cells and early senescence cells, while reducing the number of late senescence cells (Zonari et al., 2023). Another relevant peptide is UBX0101, which has proven effective in reducing the senescence burden, especially in osteoarthritis models. This peptide acts similarly to the promising peptides tested in this study, in addition to promoting cartilage regeneration and reducing inflammation (Jeon et al., 2017). These results highlight the effectiveness of

senotherapeutic peptides in different contexts, reinforcing their potential for therapies aimed at both preventing and treating the effects of cellular aging.

Furthermore, the toxicity profile of the peptides further reinforces their therapeutic potential. Peptides C3 and C10 demonstrated biocompatibility at all testes concentrations, both before and after senescence induction. In contrast, peptides such as C13, C14, and C15 exhibited significant toxicity at higher concentrations, emphasizing the importance of identifying peptides with a favorable safety profile for clinical application. These findings align with the notion that effective senotherapeutics should maintain cellular viability while exerting their protective or modulatory effects (Zonari et al., 2022).

Moreover, the modulation of NO production by the peptides provides additional insights into their therapeutic mechanisms. Elevated NO levels are often associated with inflammation and oxidative stress, hallmark features of cellular senescence (Maldonato et al., 2023; Nousis et al., 2023). However, in the endothelial context, NO plays a key role in regulating vasoreactivity, promoting vasodilation and blood flow homeostasis (Tousoulis et al., 2012). Changes in NO production, particularly in senescence states, can impair endothelial function, exacerbating vascular dysfunction and local inflammatory processes (Han et al., 2023).

Interestingly, the increase in nitrite production observed with some peptides before senescence induction highlights their ability to stimulate cellular responses, potentially enhancing resilience to stressors such as doxorubicin. On the other hand, the stable nitrite levels observed after senescence induction suggest a regulatory effect that may preserve cellular function without exacerbating inflammatory responses. The ability of peptides C3 and C10 to maintain nitrite levels close to control values, particularly after senescence induction, suggests a dual role: mitigating the inflammatory microenvironment characteristic of senescent cells and preserving endothelial function. This regulatory effect may be particularly relevant for sustaining tissue perfusion and vascular integrity in tissues compromised by cellular senescence (Huang et al., 2022). Considering that endothelial NO has a direct impact on oxygen delivery and removal of toxic metabolites, its modulation by these peptides could represent an additional mechanism by which these compounds mitigate the deleterious effects of senescence (Scioli et al., 2020; Roth et al., 2023). Future studies should focus on elucidating the specific pathways through which these peptides

influence NO production, considering both their inflammatory function and their role in endothelial regulation, with important implications for the management of dysfunctions related to senescence (Han et al., 2023).

Although the initial results are promising, it will be essential to validate peptides C3 and C10 in future studies and optimize their specificity, potency, and stability using artificial intelligence-guided techniques. The creation of a more robust database would expand the conformational space in the MRL model, favoring the development of new peptides with better senotherapeutic activity. The efficacy of peptides C3 and C10 in mitigating senescence and their favorable safety profile highlight them as promising candidates. Future research should focus on structural optimization and the investigation of their interactions with molecular targets and pathways involved in senescence and inflammation, aiming to understand their mechanisms of action and develop more precise interventions against cellular aging.

CONCLUSION

Our data demonstrated that the C3 and C10 peptides have therapeutic potential in modulating cellular senescence in dental pulp tissue. Both peptides showed effectiveness in reducing senescence before and after its induction, with the C3 peptide standing out for its higher inhibition rate, achieving a 61% reduction when used prior to senescence induction. Furthermore, the peptides exhibited a favorable safety profile and their ability to regulate nitrite production suggests a beneficial effect on modulating the inflammatory microenvironment associated with cellular senescence. Then, peptides C3 and C10 have potential for the development of effective senotherapeutic therapies, offering a promising approach to treating conditions associated with cellular aging, with potential applications in dentin-pulp complex. The use of artificial intelligence tools in optimizing these peptides opens new perspectives for research on these new molecules. Moreover, it is necessary to explore their mechanism of action to validate them as senotherapeutics. Advances in this direction will enable the development of more effective therapies to combat the effects of cellular senescence in the context of dental pulp, making a significant contribution to dentistry.

ACKNOWLEDGMENTS

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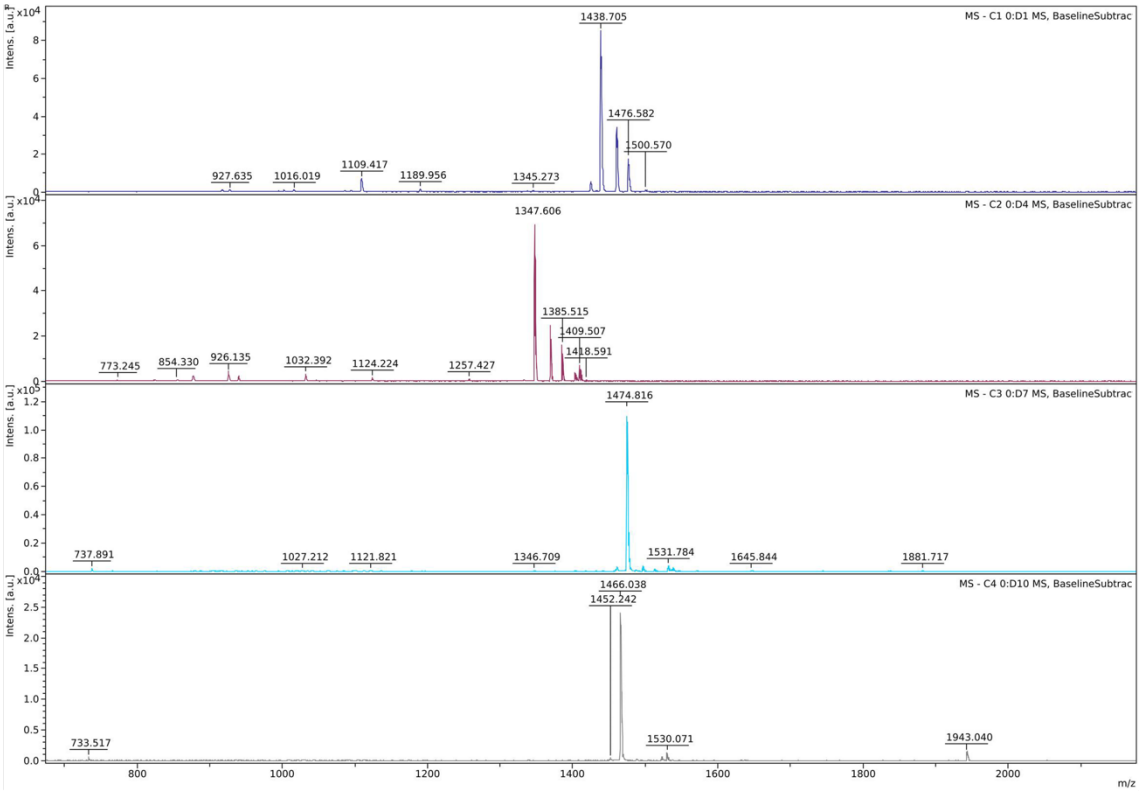
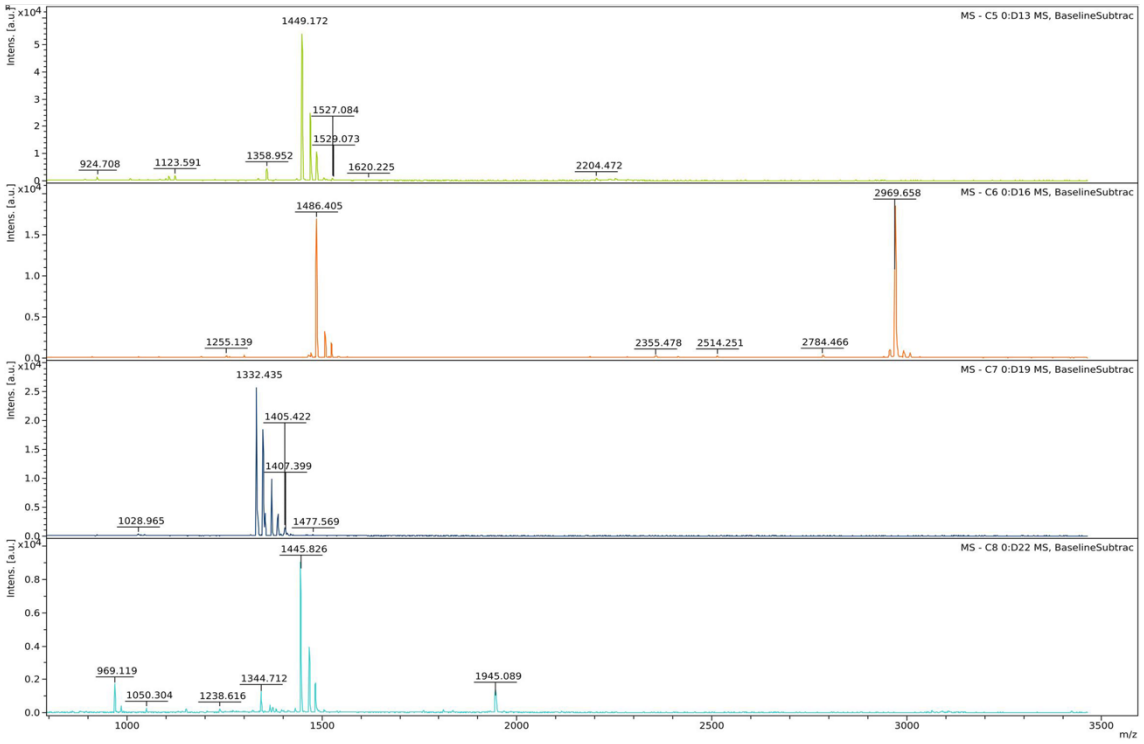
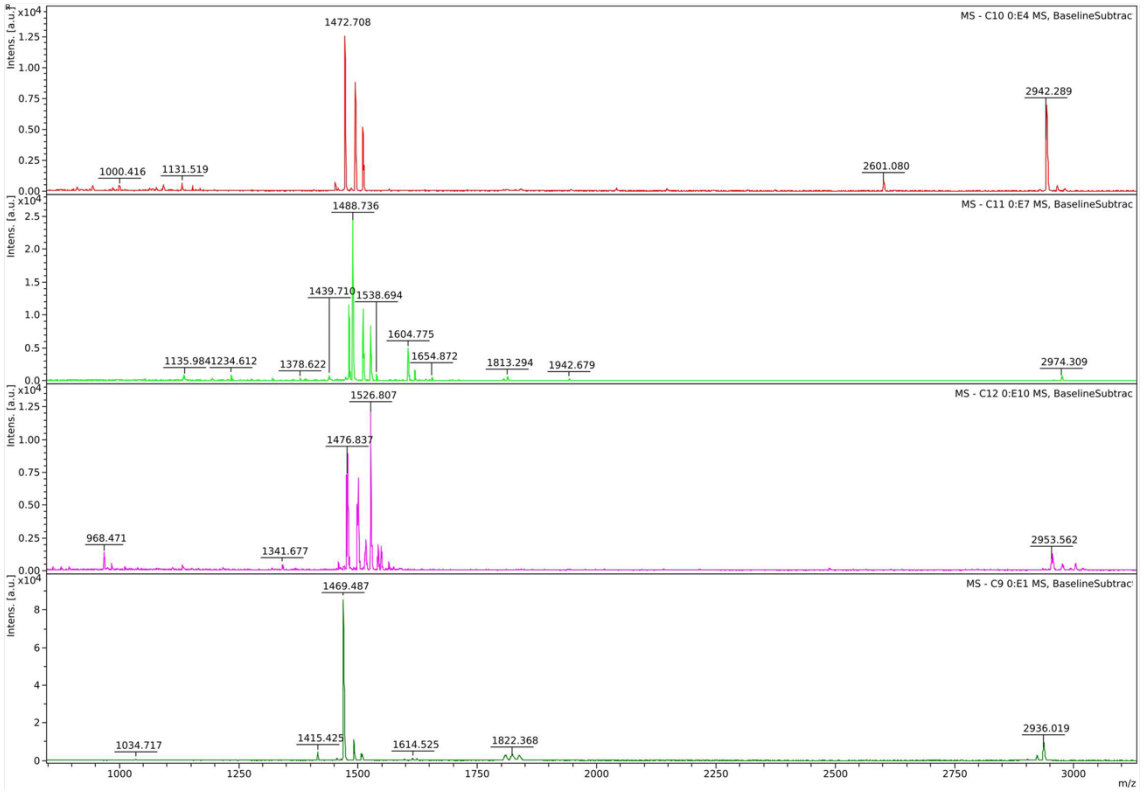
SUPPLEMENTARY INFORMATION



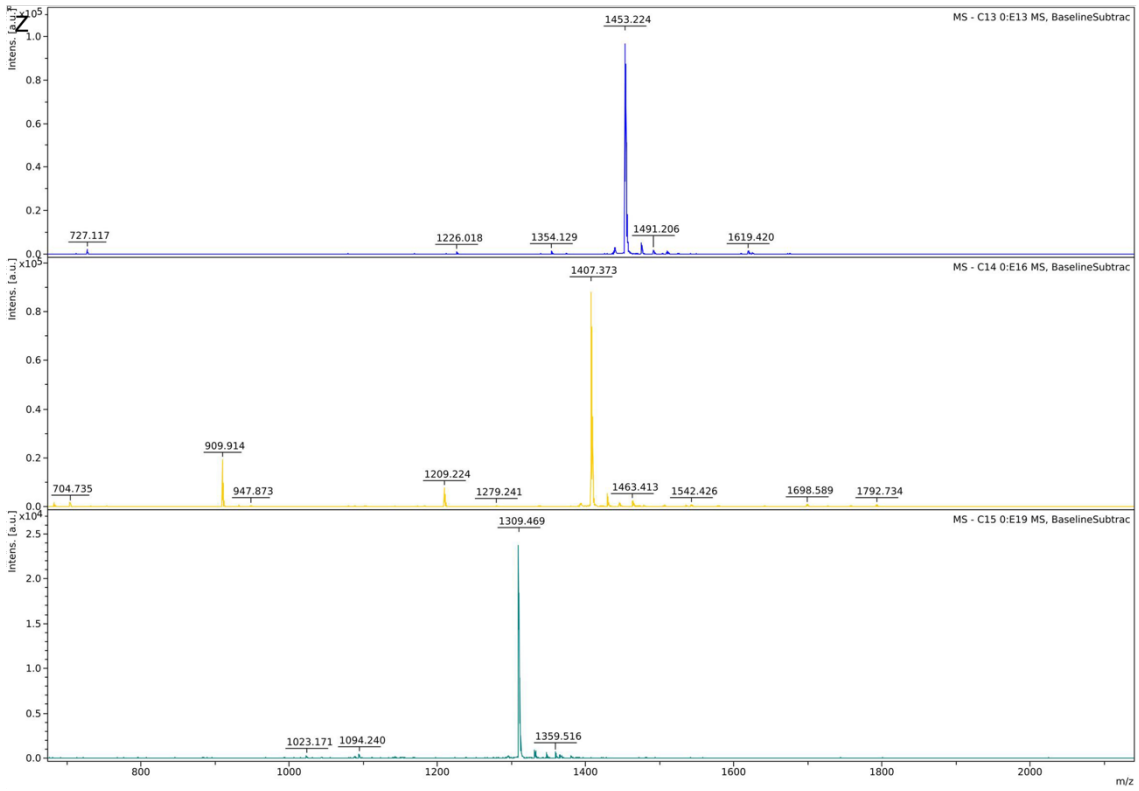
Supplementary Figure 1. Spectrum obtained by MALDI-ToF of peptides C1, C2, C3, and C4.



Supplementary Figure 2. Spectrum obtained by MALDI-ToF of peptides C5, C6, C7, and C8.



Supplementary Figure 3. Spectrum obtained by MALDI-ToF of peptides C9, C10, C11, and C12.



Supplementary Figure 4. Spectrum obtained by MALDI-ToF of peptides C13, C14 and C15.

CONSIDERAÇÕES FINAIS

Os três artigos apresentados nesta dissertação contribuem de forma significativa para o avanço das pesquisas sobre senescência celular no tecido pulpar, utilizando diversas metodologias para caracterizar esse estado e buscar alternativas terapêuticas, visando garantir a longevidade da vitalidade pulpar e melhorar o prognóstico de tratamentos endodônticos conservadores. Para o Capítulo 1, foi elaborada uma revisão de literatura detalhada com a temática da senescência no contexto odontológico, com enfoque no complexo dentino-pulpar, para isso foram utilizadas metodologias de revisões bibliométricas e de escopo. Sabemos que, esse processo tem sido tema chave na literatura ao longo dos últimos anos, especialmente devido ao impacto direto que exerce no prognóstico de tratamentos clínicos. Nossa análise revelou um crescimento expressivo nas publicações sobre o tema, tendo um aumento exponencial no número de trabalhos após 2021, principalmente sobre a senescência da polpa dentária e do ligamento periodontal.

No entanto, também demonstramos que apesar do avanço no entendimento da patogênese e do diagnóstico, ainda há uma lacuna significativa no que tange o desenvolvimento de estratégias terapêuticas e preventivas, que é algo praticamente inexistente na odontologia. Além disso, a predominância de estudos *in vitro* e *in vivo*, em detrimento de investigações clínicas e revisões sistemáticas, evidencia a necessidade de mais esforços acerca do tema, a fim de traduzir os achados laboratoriais em soluções clínicas eficazes. No mais, as redes bibliométricas revelaram os temas mais recorrentes na pesquisa e suas conexões, com as palavras-chave mais prevalentes sendo: célula, senescência e senescência celular. Concluímos que, a ocorrência dessas palavras-chave ao longo das décadas, evidencia o crescimento do interesse na pesquisa sobre o papel da senescência celular em tecidos importantes como a polpa, além de contextos odontológicos mais amplos.

No mais, este capítulo visou explorar o impacto de materiais odontológicos amplamente utilizados, como adesivos dentinários, resinas compostas e clareadores dentais, que liberam substâncias potencialmente tóxicas capazes de induzir estresse oxidativo, inflamação e danos genéticos. Com isso, esses fatores não apenas

comprometem a integridade dos tecidos dentários, mas também podem acelerar a senescência celular, influenciando diretamente o sucesso de tratamentos conservadores e a saúde bucal a longo prazo. Adicionalmente, investigamos estratégias que tem o intuito de mitigar esse processo, como senolíticos e senomórficos, drogas capazes de modular a senescência. Essas terapias têm sido difundidas em várias áreas, especialmente na medicina regenerativa, tendo demonstrado potencial para melhorar a resposta dos tecidos durante o envelhecimento celular.

Já o Capítulo 2, trás os resultados de um estudo i*n vitro* que investigou o impacto da senescência celular nas capacidades de resposta imune e regeneração do tecido pulpar, em condições de homeostasia e sob estímulo inflamatório. Sendo assim, buscamos evidenciar a relevância desse processo e como ele atua diretamente no insucesso de tratamentos endodônticos conservadores. Para isso, foi realizada a indução da senescência em células pulpares humanas, através do tratamento com doxorrubicina, o que nos permitiu uma análise aprofundada das alterações funcionais e morfológicas associadas a esse estado celular. Assim, observamos que as células pulpares senescentes apresentaram significativa redução na viabilidade, capacidade migratória e proliferativa, além de alterações morfológicas características, como aumento no tamanho celular e diminuição das extensões celulares. Esses achados são consistentes com o que vimos em outros trabalhos, evidenciando um declínio das funções celulares característico desse estado.

Ademais, a resposta imunoinflamatória das células senescentes mostrou-se exacerbada, com aumento na expressão de citocinas pró-inflamatórias como TNF-α e IL-6, além de maior expressão de IDO, indicando um microambiente inflamatório crônico e comprometimento na capacidade de resolução inflamatória. Por outro lado, a expressão reduzida de TGF-β1 e uma expressão inalterada de IL-10 em células senescentes, sugere uma diminuição na capacidade regenerativa da polpa dentária, afetando negativamente o potencial de reparo diante de lesões ou infecções. Esses resultados corroboram com a literatura existente sobre o impacto da senescência celular em diferentes tipos de tecidos, reforçando a necessidade de considerar esse estado celular na prática clínica endodôntica.

Assim, este estudo contribui significativamente para a compreensão dos desafios associados a senescência celular da polpa dentária, especialmente em

cenários inflamatórios. Os achados sugerem que a senescência celular é um fator limitante para a eficácia de tratamentos endodônticos conservadores e regenerativos, destacando a importância de pesquisas voltadas para a identificação e aplicação de peptídeos senoterapêuticos. Com isso, tais peptídeos poderiam atuar na reversão ou atenuação dos efeitos da senescência, potencializando a resposta regenerativa e imunológica da polpa dentária. Portanto, a integração de estratégias terapêuticas que considerem o estado senescente das células pulpares pode ser crucial para o desenvolvimento de abordagens mais eficazes, especialmente em pacientes mais velhos ou com polpas comprometidas.

Por fim, o Capítulo 3 deste trabalho explorou o potencial de uma biblioteca de peptídeos antimicrobianos, conhecidos por sua ação promíscua, projetados por inteligência artificial, na mitigação da senescência em células pulpares. Chegamos assim, aos peptídeos C3 e C10, que se destacaram por sua capacidade de inibir a senescência antes de sua indução e modular o fenômeno já estabelecido, sugerindo um perfil terapêutico versátil e promissor. Além disso, a utilização de ferramentas computacionais avançadas para o design desses peptídeos demonstrou o potencial da IA na descoberta de novas terapias para disfunções celulares associadas ao envelhecimento. Ademais, a biocompatibilidade dos peptídeos C3 e C10, juntamente com sua capacidade de regular a produção de óxido nítrico, reforça sua relevância como potenciais senoterapêuticos. Estes resultados estabelecem uma base sólida para futuros estudos focados na otimização estrutural desses peptídeos e na elucidação de seus mecanismos de ação, visando o desenvolvimento de intervenções mais precisas e eficazes contra o envelhecimento celular.

Em conclusão, a integração das evidências apresentadas nestes três artigos amplia significativamente o entendimento sobre a senescência celular no tecido pulpar, destacando tanto seus impactos adversos na resposta imune e regenerativa quanto o potencial de novas abordagens terapêuticas. A combinação de análises bibliométricas e experimentações *in vitro*, nos permitiu mapear lacunas críticas na literatura e demonstrar a importância de estratégias inovadoras, como o uso de peptídeos senoterapêuticos projetados por inteligência artificial. Esses resultados não apenas reforçam a necessidade de considerar a senescência celular na prática clínica, mas também apontam caminhos promissores para o desenvolvimento de tratamentos personalizados que preservem a vitalidade pulpar e melhorem o prognóstico de

terapias conservadoras. Em suma, o avanço dessas investigações, será fundamental para traduzir essas descobertas em soluções clínicas eficazes, com potencial de beneficiar especialmente pacientes com maior comprometimento pulpar ou em processos de envelhecimento.

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ANEXOS

Anexo 1. Parecer consubstanciado de aprovação do projeto no CEP/UnB (primeira e última página do parecer)

FACULDADE DE CIÊNCIAS DA SAÚDE DA UNIVERSIDADE DE BRASÍLIA - UNB

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da resposta imune em células pulpares senescentes e não senescentes e a busca por peptídeos com ação anti-senescência

Pesquisador: Johnny Carvalho da Silva

Área Temática:

Versão: 2

CAAE: 75393923.1.0000.0030

Instituição Proponente: Programa de Pós-graduação em Ciências da Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 6.626.079

Apresentação do Projeto:

Conforme documento "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_2219258.pdf", postado em 05/12/2023:

"Resumo:

A senescência celular, é caracterizada pela interrupção irreversível do ciclo celular, acompanhada de alterações morfológicas, metabólicas, lisossomais e dos fatores de secreção. Embora tenha evoluído para limitar a proliferação de células velhas ou danificadas, a senescência contribui para o envelhecimento, resultando em doenças relacionadas à idade e diminuição das funções celulares. Essas alterações afetam as células da polpa dentária, prejudicando suas funções defensivas e sensoriais e o sucesso dos tratamentos endodônticos conservadores. Para o estudo, serão cultivadas células pulpares obtidas de terceiros molares íntegros extraídos de pacientes adultos. As células serão tratadas com doxorrubicina para induzir a senescência, e o estado será confirmado por coloração de -galactosidase. Após a indução, uma resposta inflamatória simulada in vitro será realizada com estímulos de lipopolissacarídeo (LPS) e interferongama (IFN-). Em seguida, serão avaliadas as alterações da senescência na resposta imune, através da análise dos genes das citocinas pró-inflamatórias: fator de necrose tumoral alfa (TNF-), interleucina 6 (IL-6), e citocinas anti-inflamatórias fator de crescimento transformador beta 1 (TGF-1) e interleucina 10 (IL-10) por PCR.

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Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BRASILIA, 27 de Janeiro de 2024

Assinado por:
Cristiane Tomaz Rocha
(Coordenador(a))

Anexo 2. Trabalhos em colaboração publicados durante o período de mestrado.

Artigo 1: *Antibiofilm, regenerative and bone homeostasis potential of the synergistic association of synoeca-MP peptide with chlorhexidine in oral cavity opportunistic infections*, publicado na revista *Archives of Oral Biology* (IF: 2.2, A1 CAPES)

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Antibiofilm, regenerative and bone homeostasis potential of the synergistic association of synoeca-MP peptide with chlorhexidine in oral cavity opportunistic infections

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ABSTRACT

Objective: Synoeca-MP is an antimicrobial peptide that belongs to the class of defense peptides, known for their antimicrobial and immunomodulatory properties. To evaluate *in vitro* the association between synoeca-MP peptide and chlorhexidine, regarding their antimicrobial and antibiofilm activities, saliva stability, effect on tissue repair, bone resorption processes, and mineralized matrix formation.
Design: Initially, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiofilm concentration were determined. The synergism and degradation of synoeca-MP and chlorhexidine in human saliva were assessed. Furthermore, biocompatibility was evaluated using MTT assays, hemolytic assays, and proliferation and migration assays of periodontal ligament cells. Finally, bone homeostasis was evaluated through osteoclastogenesis assays, alkaline phosphatase determination, and mineralized matrix formation assay with SaOs-2 and ligament cells.
Results: The antimicrobial and antibiofilm activity against the tested microorganisms was confirmed. Low synergistic concentrations of the synoeca-MP and chlorhexidine combination inhibited tested microorganisms. The association of these molecules remained stable in healthy saliva. Nevertheless, it degraded as the severity of periodontal disease increased. Additionally, lower synergistic concentrations of the combination were not cytotoxic to human cells, promoted the proliferation and migration of ligament cells, inhibited osteoclastogenesis, and increased mineral matrix formation of ligament cells and SaOs-2.
Conclusions: Synoeca-MP and chlorhexidine combination shows potential for oral diseases treatment, as evidenced by its antimicrobial activity, regenerative potential, saliva stability, and bone homeostasis. It may be particularly effective for opportunistic oral infections and in conjunction with mechanical therapy.

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Anexo 3. Trabalhos em colaboração em fase de submissão, elaborados durante o período de mestrado.

Artigo 1: *Antimicrobial, Toxicity, and Cellular Interactions of Bioceramic Sealers in Periodontal Ligament: In Vitro Insights*, em preparação final para submissão na revista *International Endodontic Journal* (IF: 5.4, A1 CAPES)

Antimicrobial, Toxicity, and Cellular Interactions of Bioceramic Sealers in Periodontal Ligament: *In Vitro* Insights

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Running title: BIOCERAMIC SEALERS AND PERIODONTAL LIGAMENT CELLS.

Keywords: Antimicrobial, bioceramics, biocompatibility, toxicity, bioactivity.

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Artigo 2: *MTA Repair HP and Bio C Repair: Assessment of Antimicrobial, Cytotoxic, Migration and Cytokine Expression*, em preparação final para submissão na revista *International Endodontic Journal* (IF: 5.4, A1 CAPES)

MTA REPAIR HP AND BIO C REPAIR: ASSESSMENT OF ANTIMICROBIAL, CYTOTOXIC, MIGRATION AND CYTOKINE EXPRESSION

Running title: ACTIVITY OF REPAIR BIOCERAMICS

Larissa Barbosa de Sousa[1], Chrislaine Caroliny Dias[2], Mayara Alves de Oliveira[1], Raquel Figuerêdo Ramos[1], Elizabete Cristina Iseke Bispo[3], Johnny Carvalho da Silva[1], Rosiane Andrade Costa[5], Felipe Saldanha Araújo[3], Taia Maria Berto Rezende[1,4,5,6].

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Artigo 3: *Endodontic therapy in older adults patients: a narrative review*, em preparação final para submissão na revista *Gerodontology* (IF: 2.0, A2 CAPES)

Endodontic therapy in older adults patients: a narrative review

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Short Title: Endodontic therapy in older adults patients

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