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Bioengineering and inflammatory processes in oral regenerative medicine

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Orientador: Prof. Ricardo de Souza Magini, Dr.
Coorientador: Profª. Ariadne Cristiane Cabral da Cruz, Drª.

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Mariane Beatriz Sordi

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Odontologia.

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Prof. Ricardo de Souza Magini, Dr.
Orientador

Florianópolis, 2022

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This is a document that summarizes one journey of many years of postgraduation. There are so many people that crossed my way and impacted me positively that I would have to write pages endlessly. Although not mentioned them herein, I remember them all. Therefore, I will write and thank those that potentially will read this section of the document.

First and foremost, I would like to thank **my lovely family**. I think that every person that had the occasion to talk to me for more than half an hour realizes how important my family is to me. I have a mother, a father, and three sisters that I love with all my heart. They support me in every way and give me advice when I need it. The connection we have is so strong and I know I can count on them for the rest of my life. I want you to know that I love you boundlessly and I thank the universe for giving me the best family I could ever have.

Second, I thank my professors and mentors. Again, many mentors crossed my journey giving me sometimes small advice, sometimes great lessons. From the clinic, the laboratory, to the life. Not always positively, but this is what makes us decide who and how we want/should behave in life. In this sense, the first name I want to cite is Prof. **Ariadne Cristiane Cabral da Cruz**. The good life put this amazing professor on my journey when I did not know how to move forward and I was in such a desperate moment. At that time, she was preparing herself to go into the exams that led to her current professorial position at the Federal University of Santa Catarina. Without properly knowing me, she listened to my claims and helped me both personally and professionally. She gave me hope and a way to continue my studies. Today, more than a mentor/mentee relationship, we have an honest friendship, and we know we can count on each other beyond academia. Ariadne, I thank you so much for all the support, you are such an amazing human being and I extend my gratitude to your husband and your sons.

My Brazilian mentor, Prof. **Ricardo de Souza Magini,** is literally a mentor for my life and somehow a therapist. I always trusted his advice, because I know he could see me behind any word I would be able to say. And I felt this when he first interviewed me and made me go into tears. Even not knowing me so closely, you were able to tell how I was feeling. From that day on, I knew I have chosen the right mentor for my postgraduation studies. It was you, my dear Prof. Magini, who suggested I should go to King's College London. At the time of this decision, I was not sure which way I should take, so I decided to follow your recommendation and I could not imagine something better. Magini, I thank you for leading and guiding me on this journey. Thanks for receiving my calls, thanks for every opportunity that you opened to me, but, most importantly, thanks for seeing me.

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Over my postgraduation years, I had the opportunity to study abroad two times. My first journey was then to London. Even before my master's, I had the desire to work and study stem cells. It was not always easy on this cell biology journey since a few years back we did not have a cell culture lab for our program at the Federal University of Santa Catarina. With some efforts and partnerships, we managed to have our cell culture laboratory which is now located in the Centre of Biologic Sciences of the Federal University of Santa Catarina. Regardless, what a huge opportunity for me was to be able to join the Centre for Craniofacial and Regenerative Biology at King's College London, especially under the leadership of the renowned Prof. **Paul Sharpe**. I remember seeing his picture on books and in lectures and then suddenly I was joining his team. I remember training my words for our first meeting, but what a kind person he always was to me. I also remember him checking on me, asking if it was all right and if I was learning something, always positive and supportive. Unfortunately, the COVID-19 pandemic disrupted my work, which I had to leave unfinished just when I was finally starting to move nicely through the new experiments. I did not have the chance to properly say goodbye to Paul and my lovely colleagues from the lab. Nevertheless, that was a challenging time of transition in my career, moving from mostly a clinical approach to the biological fundamentals for scientific research. Dear Prof. Paul, I thank you for introducing me to this new world and I do not forget you saying, "Keep smiling, Mari" when I had to leave.

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More recently I had the opportunity to join the Oral Biology Lab, which head is Prof. **Reinhard Gruber**, as an Osteology Foundation scholar. Less planned than my first experience abroad, I did not know exactly what to expect from this new job; but I knew Prof. Gruber before, and I had not only my own admiration for his work and personality but also great recommendations from other colleagues that were able to be his mentees before. Then, a few months after our first interview and after all the visa-related bureaucracy, especially during pandemic times, I was joining his group. Our first contact was the summer lunch in his back garden, where I got to meet his family and my new lab colleagues. It was a great introduction to a group of amazing and friendly people from different cultures. Dear Prof. Gruber, thank you so much for all your time and attention. Thanks for being so kind and preoccupied with our wellbeing, happiness, culture, and future. Thank you for being so close to all of us from the lab and introducing Ulli and your kids to our life. You have a beautiful family that we know you are proud of. You are much more that a mentor and I am continually grateful for you choosing me for this job.

I am glad to have the above-mentioned names as the committee for my PhD defence day. Please know that you were carefully chosen not only because of your professional expertise but especially because of the affection and gratitude I have for you.

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and now, I am post graduating by holding a doctoral degree. This university, naturally, defined the professional I am today; but further from the professional aspect, this public and open university broadened my barriers and shaped my personality to become a humanistic and critical mind. In return, I gave and give my best to shed the light on this University as I hope for better times after such depressing times related to the Brazilian government and lack of investments in education and science. I especially thank the **Centro de Ensino e Pesquisa em Implantes Dentários (CEPID)** at the Department of Dentistry and the **Laboratótio de Virologia Aplicada (LVA)** at the Department of Microbiology, Immunology, and Parasitology of the Federal University of Santa Catarina. Second, I thank the King's College London, more specifically the **Centre for Craniofacial and Regenerative Biology**, and the Medical University of Vienna, particularly the **Competence Centre of Oral Biology**, that kindly received me for my abroad experiences. Most of all, I thank all the people I was able to meet in these different environments. It is people that make every sense in life, and it is for people that we move forward. Thus, to all the people I met, all the friends I made, every moment I had, I leave my acknowledgement. This is the end of a cycle where the most emerging sentiment is gratitude.

Finally, to conclude this acknowledgement section, I leave a quote that made me reflect on my own journey and on every person that came into my life. My thankfulness goes to you all.

"In the end, I've come to believe in something I call the 'physics of the quest', a force in nature governed by the laws of gravity. The rules of quest physics goes something like this: If you're brave enough to leave behind everything familiar and comforting and set out on a truth seeking journey either internally or externally, and if you are truly willing to regard everything that happens to you on that journey as a clue and if you accept everyone you meet along the way as a teacher and if you are prepared most of all to face and forgive some of the most difficult realities about yourself, then the truth will not be witheld from you."

— Elizabeth Gilbert, Eat, Pray, Love

RESUMO

O objetivo geral deste projeto de doutorado foi propor, por meio de estudos preliminares in vitro, terapias práticas para distúrbios orais emergentes, como doença periodontal e perda de tecido ósseo. Primeiramente, foi proposto um arcabouço para regeneração do tecido ósseo por meio de incorporação de sinvastatina em uma estrutura porosa tridimensional construída com uma mistura de ácido poli(lático-co-glicólico) (PLGA) e biocerâmica (hidroxiapatita / β-tricálcio fosfato), utilizando células de dentes decíduos esfoliados humanos (SHED) para testar o potencial osteogênico do arcabouço desenvolvido. O arcabouço proposto incorporou com sucesso a sinvastatina, o que levou à manutenção da viabilidade celular e mineralização da matriz extracelular, juntamente com a expressão de marcadores de proteínas ósseas. Em segundo lugar, foi desenvolvido um arcabouço para regeneração do tecido ósseo, incorporando o hormônio da paratireoide (PTH) na estrutura do vidro bioativo mesoporoso que foi misturado em uma estrutura de hidrogel e testou-se a capacidade de osteoindução dos arcabouços em SHED. O arcabouço demonstrou morfologia e propriedades físico-químicas, bioatividade, biocompatibilidade e capacidade de osteoindução para ser utilizado na engenharia de tecido ósseo. Portanto, os arcabouços aqui apresentados são recomendados para futuros estudos sobre bioimpressão para regeneração do tecido ósseo. Em terceiro lugar, foi proposta a aplicação do derivado da matriz do esmalte (EMD) como um produto anti-inflamatório capaz de diminuir a piroptose mediada por LPS, uma reação inflamatória exacerbada que ocorre em resposta a fatores de virulência e leva à destruição tecidual. O derivado da matriz do esmalte reduziu os fatores de piroptose em macrófagos, sugerindo uma possível aplicação clínica deste produto para reduzir a piroptose em desordens periodontais. No geral, o objetivo principal deste projeto de doutorado foi alcançado ao avaliar diferentes abordagens e terapias in vitro para distúrbios periodontais e peri-implantares, bem como propor arcabouços bioativos para engenharia de tecidos ósseos.

Palavras-chave: Regeneração óssea. Arcabouços. Células-tronco mesenquimais. Doença periodontal. Periimplantite. Piroptose.

RESUMO EXPANDIDO

Introdução

A área da ciência que estuda a aplicação de arcabouços bioativos para regeneração de tecidos é conhecida como bioengenharia. Pesquisas com foco no desenvolvimento de arcabouços bioativos seguros para fins de regeneração óssea ainda são necessárias para melhorar a previsibilidade do ganho ósseo em cirurgias maxilofaciais e, portanto, a relevância do presente projeto de tese. Nesse sentido, este projeto de tese propôs dois tipos distintos de arcabouços, aplicando polímeros sintéticos ou hidrogéis, juntamente com moléculas sinalizadoras para a diferenciação osteogênica de células-tronco de origem dentária. Além disso, discutiu-se os motivos que impedem a aplicação de arcabouços bioativos na prática clínica.

Uma das causas da perda dentária, com consequente perda óssea, é a doença periodontal. Considerando os prejuízos causado pela piroptose nos distúrbios periodontais, encontrar formas de inibir ou reduzir os processos catabólicos da piroptose traz perspectivas para terapias periodontais. Nesse sentido, este projeto de tese propôs o derivado da matriz do esmalte, um produto anti-inflamatório utilizado rotineiramente na prática clínica para regeneração de tecidos moles como um produto potencial para reduzir os danos periodontais e/ou peri-implantares. Além disso, discutiu-se sobre os fatores de virulência que podem ser terapeuticamente almejados para diminuir a inflamação exacerbada relacionada à piroptose é importante para trazer insights para pesquisadores e clínicos.

Objetivos

O objetivo geral deste projeto de doutorado foi propor, por meio de estudos preliminares in vitro, terapias práticas para distúrbios bucais emergentes, como doença periodontal e perda de tecido ósseo. Os objetivos específicos foram: 1) Propor um arcabouço para regeneração de tecido ósseo incorporando sinvastatina em uma estrutura porosa tridimensional construída com uma mistura de polímero de ácido poli(lático-co-glicólico) (PLGA) e biocerâmica (hidroxiapatita/β-fosfato tricálcico), usando células de dentes humanos decíduos esfoliados (SHED) para testar o potencial osteogênico in vitro do arcabouço projetado para este fim; 2) Propor um arcabouço para a regeneração de tecido ósseo, incorporando o paratormônio (PTH) em uma estrutura de vidro bioativo mesoporoso, o qual foi posteriormente adicionado em uma construção porosa de hidrogel, também utilizando células de dentes decíduos esfoliados (SHED) para testar o potencial osteogênico in vitro do arcabouço; 3) Propor o derivado da matriz do esmalte (EMD) como um produto anti-inflamatório capaz de diminuir fatores relacionados com a piroptose mediada por lipopolisacarídeos (LPS) em macrófagos.

Metodologia

Primeiramente, foi proposto um arcabouço para regeneração do tecido ósseo por meio de incorporação de sinvastatina em uma estrutura porosa tridimensional construída com uma mistura de PLGA e biocerâmica (hidroxiapatita / β-tricálcio fosfato), utilizando SHED para testar o potencial osteogênico do arcabouço desenvolvido. Em segundo lugar, foi desenvolvido um arcabouço para regeneração do tecido ósseo, incorporando PTH na estrutura do vidro bioativo mesoporoso que foi misturado em uma estrutura de hidrogel e testou-se a capacidade de osteoindução dos arcabouços em SHED. Em terceiro lugar, foi proposta a aplicação do EMD como um produto anti-inflamatório capaz de diminuir a piroptose mediada por LPS, uma reação inflamatória exacerbada que ocorre em resposta a fatores de virulência e leva à destruição tecidual.

Resultados e Discussão

O arcabouço de PLGA e biocerâmica proposto incorporou com sucesso a sinvastatina, o que levou à manutenção da viabilidade celular e mineralização da matriz extracelular, juntamente com a expressão de marcadores de proteínas ósseas. O arcabouço de hidrogel com vidro bioativo incorporando PTH demonstrou morfologia e propriedades físico-químicas, bioatividade, biocompatibilidade e capacidade de mineralização da matriz extracelular, sendo compatível para utilização na engenharia de tecido ósseo. Portanto, os arcabouços aqui apresentados são recomendados para futuros estudos sobre bioimpressão para regeneração do tecido ósseo. O EMD reduziu os fatores de piroptose em macrófagos, sugerindo uma possível aplicação clínica deste produto para reduzir a piroptose em desordens periodontais.

Considerações finais

No geral, o objetivo principal deste projeto de doutorado foi alcançado ao avaliar diferentes abordagens e terapias in vitro para distúrbios periodontais e peri-implantares, bem como propor arcabouços bioativos para engenharia de tecidos ósseos. O presente projeto de tese permitiu tirar duas conclusões principais a partir dos objetivos principais deste estudo e abriu espaço para pesquisas futuras.

Inicialmente, como objetivo do presente projeto de tese de doutoramento, abordou-se os arcabouços projetados para engenharia de tecido ósseo. Arcabouços tridimensionais para regeneração óssea são difíceis de serem produzidos, mas são promissores para aplicações clínicas considerando as tecnologias emergentes, especialmente a bioimpressão tridimensional, que permite uma maneira reprodutível de fabricar arcabouços juntamente com células e moléculas sinalizadoras. Profusa pesquisa científica foi publicada no campo das estratégias de aumento do tecido ósseo; no entanto, poucas estão obtendo sucesso na aplicação clínica a longo prazo. Para além da dificuldade de regenerar um tecido complexo como o osso, a falta de sucesso na aplicação clínica se deve provavelmente à enorme lacuna entre a pesquisas *in vitro* e as condições *in vivo*, principalmente no que diz respeito ao comportamento celular. Portanto, é imperativo discutir os motivos que impedem que todos os esforços feitos para desenvolver arcabouços para engenharia de tecidos ósseos sejam translacionados para a aplicação clínica. Uma melhor compreensão das transições celulares de um estado quiescente e indiferenciado até o ponto em que elas respondem a estímulos que permitem que elas se diferenciem na linhagem desejada é obrigatória. Isso ocorre porque as células são a chave para a medicina regenerativa e para as terapias de engenharia de tecidos. Além disso, a compreensão das transições celulares fornecerá *insights* não apenas para a regeneração do tecido ósseo craniofacial, mas também para outros campos em todo o organismo humano e, além disso, dará respostas às demandas não atendidas da biologia celular.

Paralelamente, como objetivo deste projeto de tese, abordou-se a piroptose, a qual é uma via de doenças inflamatórias que foram recentemente levantadas como uma preocupação na odontologia, especialmente nos distúrbios periodontais. Contudo, alguns compostos anti-inflamatórios são promissores como substâncias potenciais para reduzir a exacerbação da piroptose nos tecidos periodontais. Uma vez que há fortes evidências *in vitro* de que a matriz derivada do esmalte possui propriedades anti-inflamatórias, foi proposto e reportado com sucesso que tal matriz tem potencial para reduzir os fatores relacionados à piroptose em macrófagos inflamatórios. No entanto, este foi um achado de dados muito preliminar em linhagens de células murinas. Outras formas de induzir piroptose são necessárias, uma vez que o presente modelo não conseguiu provocar totalmente a cascata de piroptose. Além disso, a introdução de outras linhagens celulares orais humanas, como células epiteliais e fibroblásticas, são necessárias antes da translação para pesquisa *in* vivo a partir dos resultados encontrados. No entanto, foi a primeira vez que a matriz derivada do esmalte foi associada à piroptose em

uma abordagem de biologia oral. Isso fornece muitos *insights* para novas pesquisas e abre espaço para a experimentação de outras substâncias que podem ser aplicadas para reduzir ou inibir a exacerbação da piroptose. Além disso, entender a piroptose em uma abordagem do ambiente oral é fundamental para encontrar soluções clínicas para distúrbios periodontais e peri-implantares.

Palavras-chave: Regeneração óssea. Arcabouços. Células-tronco mesenquimais. Doença periodontal. Periimplantite. Piroptose.

ABSTRACT

The overall objective of this doctoral project was to propose, through *in vitro* preliminary studies, practical therapies for emergent oral disorders, such as periodontal disease and bone tissue loss. First, it was proposed a scaffold for bone tissue regeneration by embedding simvastatin into a three-dimensional porous structure built with a blend of poly(lactic-co-glycolic) acid (PLGA) and bioceramics (hydroxyapatite / β-tricalcium phosphate), using cells from human exfoliated deciduous teeth (SHED) to test the osteogenic potential of the designed scaffold. The proposed scaffold successfully incorporated simvastatin, which led to cell viability maintenance and mineralization of the extracellular matrix, together with the expression of bone protein markers. Second, it was projected a scaffold for bone tissue regeneration by incorporating parathyroid hormone into the structure of mesoporous bioactive glass that was blended into a hydrogel construct and tested the scaffolds' osteoinduction capacity in SHED. The scaffold demonstrated morphology and physicochemical properties, bioactivity, biocompatibility, and osteoinduction capacity to be used in bone tissue engineering. Therefore, the herein presented scaffolds are recommended for future studies on bioprinting for bone tissue regeneration. Third, it was proposed the application of enamel matrix derivative as an anti-inflammatory product capable of lowering the LPS-mediated pyroptosis, an exacerbated inflammatory reaction that occurs in response to virulence factors and leads to tissue destruction. The enamel matrix derivative dampened pyroptosis factors in macrophages, suggesting a possible clinical application of this product to reduce pyroptosis in periodontal disorders. Overall, the main objective of this doctoral project was achieved by evaluating different approaches and therapies *in vitro* for periodontal and peri-implantar disorders as well as proposing bioactive scaffolds for bone tissue engineering.

Keywords: Bone regeneration. Scaffolds. Mesenchymal stem cells. Periodontal disease. Peri-implantitis. Pyroptosis.

SUMMARY

1 INTRODUCTION

1.1 PART 1 - SCAFFOLDS FOR BONE TISSUE REGENERATION

The loss of bone dimensions is very common in individuals who have suffered from tooth loss or degenerative diseases. In general, when these individuals seek to restore health, function, and aesthetics through rehabilitation treatment, it is difficult to install dental implants due to bone dimension reductions. Then, it is necessary to clinically apply bone grafts. However, even nowadays, the predictability of bone augmentation is reduced due to the difficulty in regenerating a dynamic and vascularized tissue such as bone (CIAPETTI; GRANCHI; BALDINI, 2012; KUSS et al., 2018; YANG; ZHANG; GANGOLLI, 2014).

Due to its biological and mechanical properties, bone tissue requires intricate strategies to allow the reconstitution of its structure and function. Bone healing is a signalling cascade process, involving cells secreting cytokines, growth factors, and pro-inflammatory factors in the defect site and, subsequently, recruiting surrounding stem cells to migrate, proliferate, and differentiate into bone-forming cells (BAI et al., 2018; LEE; SILVA; MOONEY, 2011). Bone tissue engineering is an interdisciplinary science that applies the principles of biology and engineering to develop biomaterials to substitute, restore, maintain, or improve the functions of the bone (LIU et al., 2016; ROSA; DE OLIVEIRA; BELOTI, 2008). The minimum requirements for an effective bone tissue engineering strategy comprise (1) an adequate number of undifferentiated cells, (2) a scaffold capable of supporting these cells and further allowing neovascularization, and (3) signalling molecules able to induce undifferentiated stem/precursor cells to differentiate into bone-forming cells (CIAPETTI; GRANCHI; BALDINI, 2012; FAHIMIPOUR et al., 2019; LIU et al., 2016; MCMILLAN et al., 2018; ROSA; DE OLIVEIRA; BELOTI, 2008). Even in a controlled *in vitro* environment, the acquisition of such requirements for regenerative purposes is complex. Nevertheless, these three essential requirements for bone tissue engineering are necessary to give an insight of whether a new bioactive material may be promising for a possible clinical application and therefore important as an initial step in the search for alternatives for bone graft therapies. Furthermore, the knowledge of craniofacial bone tissue engineering can be applied to other tissues throughout the human body and help to move regenerative medicine forward.

Mesenchymal stem cells (MSCs) represent an attractive route for regenerative medicine applications due to their ability to differentiate into restricted but multiple cell lines. The

advantage of using MSCs is the ease of acquisition since these cells can be obtained from several human oral tissues, such as dental pulp from permanent teeth (DPSC) (GRONTHOS et al., 2000; SEVARI et al., 2020) and dental pulp of exfoliated deciduous teeth (SHED) (MIURA et al., 2003). MSCs that can differentiate into bone-forming cells under proper stimuli have an important role in the osteogenesis (CIAPETTI; GRANCHI; BALDINI, 2012; SHEIKH; SIMA; GLOGAUER, 2015) and, therefore, are important for *in vitro* characterisation to assess the feasibility of the application of MSCs for regenerative purposes. However, it is imperative to mention that it is still unsure to what extent the *in vitro* MSCs behaviour can be reproduced clinically. While it is overwhelming the efforts made to find solutions for regenerative medicine *in vitro*, it is exciting to explore alternatives to better resemble the *in vivo* conditions. In this sense, there are means to approximate preliminary *in vitro* research to more advanced *in vivo* circumstances, such as the application of three-dimensional cell cultures and additive manufacturing with the use of bioprinting of undifferentiated cells into three-dimensional constructs. The incorporation and delivery of MSCs within a suitable biomaterial would help to maintain the delivered cells at the applied site, support the integration of the graft with the surrounding tissues, and provide an environment for cells to modulate their osteogenic differentiation (MCMILLAN et al., 2018; THORPE et al., 2018). These applications, however, require an adequate number of high-quality cells, while the scalable production of human multipotent MSCs and their derivatives under well-defined conditions is a challenge (LEI et al., 2014; LEI; SCHAFFER, 2013).

The activity of MSCs is regulated by interactions with the extracellular matrix (ECM). Thus, applying biomaterials with similar performance to ECM is recommended (CIAPETTI; GRANCHI; BALDINI, 2012). The clinical application of bioactive scaffolds that can mimic the ECM may overcome the challenges of bone reconstruction. Nevertheless, the use of scaffolds is extremely intricate, since they should ideally play the role of osteoconduction – by supporting bone-forming cells, providing nutrients, and sustaining the arrival of new blood vessels – and, if possible, act on the osteoinduction – by slowly releasing bioactive molecules that stimulate mesenchymal cells to differentiate and deposit mineralized bone matrix (BOSE; VAHABZADEH; BANDYOPADHYAY, 2013; CIAPETTI; GRANCHI; BALDINI, 2012; FAHIMIPOUR et al., 2019; KUSS et al., 2018; SEVARI et al., 2020; YANG; ZHANG; GANGOLLI, 2014). Scaffolds based on bioceramics and polymers have evolved shifting from a passive role where they are merely accepted by the body to an active role where they respond

to environmental conditions or to different types of cues generating suitable integration inside the host tissue (BENEDINI et al., 2020). Herein, while designing the scaffolds for bone tissue engineering preliminary studies, it was cautiously selected inert raw materials, such as polymers and hydrogels, that would allow the incorporation of signalling molecules and be suitable for three-dimensional bioprinting. Likewise, the addition of bioceramics, herein the hydroxyapatite, β-tricalcium phosphate, and bioactive glasses, was thought to give the cells additional information on the calcified environment of bone tissue and further mechanical support to the constructs. However, a passive scaffold can offer osteoconduction to the cells but lacks osteoinduction and should be further improved towards bioactivity.

A variety of signalling molecules have been investigated for their ability to induce and accelerate bone regeneration. However, most of them frequently fail in translational trials due to side effects resulting from the supraphysiological concentrations needed to achieve the desired repair. Growth factors are naturally occurring proteins that are an integral component in controlling cellular functions, such as migration and proliferation, and determining cell fate (BAI et al., 2018; BASTAMI et al., 2017; VENKATESAN et al., 2017). Although significant osteogenic potential has been evidenced by growth factors, such as the bone morphogenetic protein (BMP) and platelet-derived growth factors, their clinical application is limited due to variations of their physical properties during the incorporation procedure, short half-life, immunogenicity complications, rapid distribution by body fluids, large doses required, inactivation by inflammatory cytokines, inefficient tissue formation, and clinical side effects. (BASTAMI et al., 2017; FAHIMIPOUR et al., 2019; LIU et al., 2016; RAHMAN et al., 2015; XU et al., 2001; YAO et al., 2006). Therefore, this thesis project aimed at finding alternative signalling molecules that would not affect cell viability and reduce possible inflammatory reactions or other adverse effects. In this sense, simvastatin and parathyroid hormone were previously reported for their potential to differentiate MSCs into a bone-like cell lineage (CHAN; MCCAULEY, 2013; DANG et al., 2017; FENG et al., 2020; GENTILE et al., 2016; MUNDY et al., 1999) and were thus incorporated into the designed scaffolds, thus providing additional information to the undifferentiated cells regarding osteoinduction.

Research focusing on the development of safe bioactive scaffolds for bone regeneration purposes is still needed to improve the predictability of bone augmentation in maxillofacial applications and therefore the relevance of the present thesis project. Moreover, it is essential to discuss what are the reasons that are preventing the application of the developed bioactive scaffolds in clinical practice.

1.2 PART 2 - INFLAMMATORY PROCESSES ON PERIODONTAL DISEASE

Periodontal disease is one of the most prevalent infectious human inflammatory diseases, and it is characterised by the inflammatory reaction and the progressive destruction of the tooth-supporting tissues (DAHLEN; BASIC; BYLUND, 2019). It is a response to years of prolonged exposure to a polymicrobial community in the gingiva and periodontal pocket (DAHLEN; BASIC; BYLUND, 2019). Periodontitis and peri-implantitis are universally agreed to begin with a breakdown in the soft tissue attachment and bone loss progression (LINDHE; MEYLE; GROUP D OF EUROPEAN WORKSHOP ON PERIODONTOLOGY, 2008; SCHWARZ et al., 2018). Consequently, methods to strengthen, maintain, or regenerate the soft tissue attachment around the tooth or the dental implant are critical for improving the sealing protection against microbial infections or endogenous danger signals (YUAN et al., 2021). Even though periodontal disorders are long ago reported, it is still a current clinical issue that significantly impacts the quality of life of individuals affected by such inflammatory disease.

The underlying pathogenesis of periodontitis/peri-implantitis is a chronic inflammation that drives downstream catabolic cellular events ultimately leading to tooth loss due to a lack of supporting tissues (KAJIYA; KURIHARA, 2021; KINANE; STATHOPOULOU; PAPAPANOU, 2017; SCHWARZ et al., 2018). There is thus a critical demand to understand the fundamental pathological mechanisms on a cellular and molecular basis to implement therapies aiming to regulate inflammation and thereby pave the way for regenerative strategies (KAJIYA; KURIHARA, 2021; KINANE; STATHOPOULOU; PAPAPANOU, 2017). Thus, understanding the pathways connecting inflammation and tissue destruction will help to develop strategies to prevent and treat periodontitis and peri-implantitis. The recent findings brought from other medical areas, such as rheumatology, are evidence that periodontitis/peri-implantitis pathogenesis is still not fully described. Such findings relate periodontal disorders with alternative inflammatory pathways, for instance, pyroptosis.

Pyroptosis is an inflammatory caspase-dependent catabolic process that is relevant for innate immunity. This process is mainly mediated by the activation of caspase-1 (CAS1) by the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing protein 3 (NLRP3) inflammasome triggered by the exposure of cells/tissues to virulence factors (YU et al., 2021). CAS1 then cleaves the gasdermin D (GSDMD), a specific pyroptosis-related factor, which is responsible for cell membrane perforation and release of interleukins-1β (IL-1β) and

-18 (IL-18) (YU et al., 2021), which in turn initiate a robust inflammatory response on the surrounding tissues (CHEN et al., 2021). Therefore, NLRP3 and CAS1 are important for bacterial clearance as part of the innate immunity; however, when overexpressed, they may lead to cellular self-destruction, inflammation, and tissue damage (SORDI et al., 2022). While this process is well-described for other inflammatory diseases such as rheumatoid arthritis and ulcerative colitis (CHADHA et al., 2020; SENDLER; MAYERLE; LERCH, 2016), just recently pyroptosis was raised as a potential pathway leading to periodontal disorders and periodontal tissue destruction.

In vitro periodontal models in pyroptosis research have focused on the NLRP3/CAS1/GSDMD-mediated pyroptosis pathway in monocytes, macrophages, and periodontal ligament cells (CECIL et al., 2017; CHEN et al., 2021; FLEETWOOD et al., 2017; ZHANG et al., 2021). NLRP3 inflammasome can react to a wide range of bacterial ligands and play a pivotal role in the pathogenesis of inflammatory diseases. Lipopolysaccharide (LPS) is a virulence factor and a strong agonist of toll-like receptors (TLR) signalling that is able to initiate the pyroptosis downstream (LIU; CUI; SHEN, 2020; ZHANG et al., 2021). LPS is produced by Gram-negative bacteria (DE ANDRADE; ALMEIDA-DA-SILVA; COUTINHO-SILVA, 2019). Thus, considering that oral diseases are mainly mediated by Gram-negative bacteria, it makes sense that LPS is in close relation to periodontal disorders (DE ANDRADE; ALMEIDA-DA-SILVA; COUTINHO-SILVA, 2019; PIHLSTROM; MICHALOWICZ; JOHNSON, 2005; SORDI et al., 2022). Consequently, NLRP3/CAS1/GSDMD-mediated pyroptosis bioassays are suitable to identify components that lower pyroptosis signalling.

Considering the impairment caused by pyroptosis on periodontal disorders, finding ways to inhibit or reduce pyroptosis downstream brings prospects for periodontal therapies. In this sense, this thesis project proposed the enamel matrix derivative, an anti-inflammatory product routinely used in the clinical practice for soft tissue regeneration (GRAZIANI et al., 2020; MYHRE et al., 2006; RAMENZONI et al., 2021; SATO et al., 2008), as a potential product to dampen pyroptosis-mediated periodontal/peri-implantar disorders. Additionally, the discussion on the virulence factors that could be therapeutically targeted to decrease exacerbated inflammation related to pyroptosis is significant to bring insights to researchers and clinicians.

1.3 OBJECTIVES

1.3.1 Main objective

The overall objective of this doctoral project was to propose, through *in vitro* preliminary studies, practical therapies for emergent oral disorders, such as periodontal disease and bone tissue loss.

1.3.2 Specific objectives

a) To propose a scaffold for bone tissue regeneration by embedding simvastatin into a three-dimensional porous structure built with a blend of poly(lactic-co-glycolic) acid polymer (PLGA) and bioceramics (hydroxyapatite / β-tricalcium phosphate), using cells from human exfoliated deciduous teeth (SHED) to test the *in vitro* osteogenic potential of the designed scaffold.
b) To review and report the new approaches for bone tissue engineering using bioactive hydrogel-based scaffolds, cell-based therapies, and three-dimensional bioprinting.
c) To propose a scaffold for bone tissue regeneration by incorporating parathyroid hormone (PTH) into the structure of mesoporous bioactive glass that was blended into a hydrogel porous construct, using cells from human exfoliated deciduous teeth (SHED) to test the *in vitro* osteogenic potential of the designed scaffold.
d) To review and discuss pyroptosis as a catabolic inflammatory mechanism occurring in the oral environment in the face of exposure to damaging/virulence factors and propose it as a target for periodontal therapies.
e) To propose enamel matrix derivative (EMD) as an anti-inflammatory product capable of lowering the *in vitro* LPS-mediated pyroptosis factors in macrophages.

2 DEVELOPMENT

The present thesis project is compiled from several pieces of research performed during the doctoral period. *In vitro* preliminary studies were performed to propose practical therapies for emergent oral disorders, such as periodontal disease and bone tissue loss. Additionally, literature reviews were carried out to delve into the research topic. Therefore, five articles were produced as a result of the main objective of this thesis project. They are presented in the next chapters of this document. The articles are in the format requirements of the Journals where they were published or submitted for publication.

2.1 ARTICLE 1- ENHANCED OSTEOINDUCTIVE CAPACITY OF POLY(LACTIC-CO-GLYCOLIC) ACID AND BIPHASIC CERAMIC SCAFFOLDS BY EMBEDDING SIMVASTATIN

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ORIGINAL ARTICLE

Enhanced osteoinductive capacity of poly(lactic-co-glycolic) acid and biphasic ceramic scaffolds by embedding simvastatin

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Abstract

Objectives This study evaluated the effect of embedding simvastatin (SIM) on the osteoinductive capacity of PLGA + HA/βTCP scaffolds in stem cells from human exfoliated deciduous teeth (SHED).

Materials and methods Scaffolds were produced by PLGA solvent dissolution, addition of HA/βTCP, solvent evaporation, and leaching of sucrose particles to impart porosity. Biphasic ceramic particles (70% HA/30% βTCP) were added to the PLGA in a 1:1 (w:w) ratio. Scaffolds with SIM received 1% (w:w) of this medication. Scaffolds were synthesized in a disc-shape and sterilized by ethylene oxide. The experimental groups were (G1) PLGA + HA/βTCP and (G2) PLGA + HA/βTCP + SIM in non-osteogenic culture medium, while (G3) SHED and (G4) MC3T3-E1 in osteogenic culture medium were the positive control groups. The release profile of SIM from scaffolds was evaluated. DNA quantification assay, alkaline phosphatase activity, osteocalcin and osteonectin proteins, extracellular calcium detection, von Kossa staining, and X-ray microtomography were performed to assess the capacity of scaffolds to induce the osteogenic differentiation of SHED.

Results The release profile of SIM followed a non-liner sustained-release rate, reaching about 40% of drug release at day 28. Additionally, G2 promoted the highest osteogenic differentiation of SHED, even when compared to the positive control groups.

Conclusions In summary, the osteoinductive capacity of poly(lactic-co-glycolic) acid and biphasic ceramic scaffolds was expressively enhanced by embedding simvastatin.

Clinical relevance Bone regeneration is still a limiting factor in the success of several approaches to oral and maxillofacial surgeries, though tissue engineering using mesenchymal stem cells, scaffolds, and osteoinductive mediators might collaborate to this topic.

Keywords Bone regeneration · Simvastatin · Mesenchymal stem cells · Scaffolds · Osteogenic differentiation

Introduction

Dimensional limitations of alveolar bone due to the resorption and remodeling after exodontia, infection, periodontal disease, or trauma may complicate, or prevent, the rehabilitation with implant-supported prostheses. Tissue engineering studies of new approaches, including scaffolds, signaling molecules, and mesenchymal stem cells (MSC), have been widely applied and promising results are obtained [1–13].

Osteoconductive scaffolds may be used as a framework to allow cell and vessel invasion, facilitating, or even accelerating, bone regeneration. In implant dentistry, scaffolds are not intended to act as a permanent bone substitute [14] but provide a temporary structure with similar properties to those of the host bone. The combination of poly(lactic-co-glycolic)

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acid (PLGA) polymer with hydroxyapatite (HA) and β-tricalcium phosphate (βTCP) ceramics synergize to generate a more favorable biomaterial. The beneficial characteristics of the individual substances, such as the release of calcium and phosphate ions from the biphasic ceramics, and the improvement of mechanical resistance to compression, adequate degradation rate [15–22], and incorporation of bioactive substances into the polymeric scaffolds, synergize to form a controlled-release system for osteoinduction [23, 24].

Drug delivery systems with osteoinductive substances may improve the performance of the scaffolds. Statins, specifically simvastatin (SIM), are potent reducers of endogenous cholesterol synthesis and are widely used to prevent coronary disease and atherosclerosis. Their effect involves an increase of the bone morphogenetic protein (BMP) expression levels in bone cells and the stimulus of new bone formation [25–29]. SIM applied alone, i.e., with no carrier, demonstrated osteogenic effect over undifferentiated cells in vitro [30–35]. However, the clinical success of SIM in bone regeneration is related to a slow and controlled release process since high local dosages induce an exacerbated inflammatory response [16, 29, 36] due to the cytotoxicity and the reduction of cholesterol production in cell membranes [7, 28]. Consequently, to reach a sustained SIM release to the applied site, a carrier (normally a three-dimensional material or scaffold) is recommended. Additionally, the use of SIM leads to a reduced risk of clinical side-effects compared to growth factors or gene therapies [30]. Therefore, this study evaluated the effect of embedding SIM on the osteoinductive capacity of PLGA + HA/βTCP scaffolds in stem cells from human exfoliated deciduous teeth (SHED).

Methods

Samples preparation

Scaffolds were produced with PLGA and biphasic ceramic composed of HA and βTCP obtained by PLGA solvent dissolution, the addition of HA/βTCP, solvent evaporation, and leaching of sucrose particles, as previously described [17, 18]. Briefly, PLGA composite was a 1:1 blend of polylactic acid (Resomer LT 706S) lactide-co-glycolide (Resomer LG 824S) (Evonik Boehringer Ing. Pharma GmbH&Co. KG, Germany). Biphasic ceramic (Genphos, Genius with HA and βTCP in the ratio 70/30, Baumer, São Paulo, Brazil) were added to the polymer blend in a 1:1 ratio. For samples containing SIM (≥ 97%, high-performance liquid chromatography (HPLC) grade, solid M = 418.57, Sigma-Aldrich, St. Louis, Missouri, USA), the drug was added to the polymer blend at 10 mg/g. Samples were sectioned into discs of 5 mm diameter and 1.5 mm height and sterilized by ethylene oxide.

SIM release

Samples were incubated in 5 mL of phosphate-buffered saline (PBS), pH 7.4, in a 37 °C and 5% CO_2 incubator chamber for up to 90 days. At different time intervals, 200 µL of the solution was collected and the absorbance was measured on a UV–vis spectrophotometer at 238 nm (TECAN, Zürich, Switzerland). The collected PBS was quantified and returned to the original solution at the end of each reading. The standard curve was obtained from known concentrations of SIM in acetonitrile and used to quantify the loaded SIM and its release from the samples. To analyze the SIM release profile, the SIM concentration was re-dimensioned by dividing the absolute amount of SIM released at time t (Mt) by the amount of SIM at infinity (M∞), which was the equilibrated concentration obtained at the end of the 90-day experimental period, since it depends not only on how much drug has been added but also on the solubility of the material. The models were evaluated based on the correlation coefficient (r^2) [17].

MSC cultures

To determine the scaffold capacity to induce the osteogenic differentiation of SHED, cell cultures were conducted using 96-well plates with 2×10^4 cells per well at 37 °C and 5% CO_2. Cell culture media for SHED were Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fischer Scientific, Waltham, USA) with 10% fetal bovine serum (FBS, Invitrogen, Thermo Fischer Scientific, Waltham, USA) (regular medium) or DMEM with 10% FBS, 1% penicillin–streptomycin (PS, Invitrogen, Thermo Fischer Scientific, Waltham, USA), 50 mM ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, USA), and 0.1 mM dexamethasone (Sigma-Aldrich, St. Louis, USA) (osteogenic medium). Alpha-Modified Minimum Essential Medium (α-MEM, Nutricell, SP, Brazil) with 10% FBS, 1% PS, 50 mM ascorbate-2-phosphate, and 10 mM β-glycerophosphate (osteogenic medium) was used for pre-osteoblasts MC3T3-E1 subclone 4 (ATCC, Virginia, USA). Two experimental groups were determined: (G1) PLGA + HA/βTCP and (G2) PLGA + HA/βTCP + SIM, applying SHED in a regular medium. The average mass of scaffolds for the experimental was 0.02 g. Additionally, two positive control groups were established: (G3) SHED and (G4) MC3T3-E1 in an osteogenic culture medium. All experiments were performed in triplicate at predetermined experimental times.

DNA quantification assay

On days 3, 7, 14, 21, and 28, cell proliferation was assessed through the content of DNA in the groups using Quant-iT TM PicoGreen® dsDNA Reagent (P7589, Invitrogen, Thermo Fisher Scientific, CA, USA). Analyses were performed according to Sordi et al. (2021) [37] and readings were carried out on a fluorescence spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA) at 480/520 nm (Ex/Em).

Osteogenic differentiation assays

Alkaline phosphatase (ALP) activity was measured using the Alkaline Phosphatase Fluorimetric Assay Kit (Abnova, Taipei, Taiwan). Analyses were performed according to the manufacturer's recommendations and readings were performed on a fluorescence spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA) at 360/440 nm (Ex/Em). Experimental times were on days 3, 7, and 14. Data were normalized against DNA content.

Extracellular calcium quantification was measured using QuantiChrom Calcium Assay Kit (Bioassay Systems, California, USA). Analyses were performed according to the manufacturer's recommendations and absorbance was recorded using a spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA) at 612 nm. Calcium quantifications were performed on days 7, 14, 21, and 28.

Quantification of osteocalcin and osteonectin proteins was measured using the Quantikine ELISA Human Osteocalcin Immunoassay and Human SPARC Immunoassay (R&D Systems, Minnesota, USA), respectively [38, 39]. Analyses were performed according to the manufacturer's recommendations at 450 nm with wavelength correction set to 540 nm (SpectraMax M2e, Molecular Devices, CA, USA). Evaluations were performed on days 14 and 21.

Nodules of mineralization were marked using von Kossa staining. For that, cells were fixed with 4% paraformaldehyde for 60 min at room temperature. Then, cells were washed with ultrapure water, covered with 1% silver nitrate solution, and incubated for 30 min, protected from light. Counterstaining with 0.1% eosin solution in ethanol was performed and the results were observed and photographed from a light microscope [40]. Analyses were performed on day 28.

X-ray microtomography (µCT)

Images of scaffold microstructure were obtained using high-resolution three-dimensional X-ray microtomography (Versa XRM-500, ZEISS/Xradia, Oberkochen, Germany). The scanning conditions were the same for all samples: X-ray tube at 50 kV/4 W, no filter for beam hardening effect, 0.4 × optical lens, 0.225° angular step, and 3 s exposure time. The resulting images had a spatial resolution (voxel size) of 9.40–9.44 µm [17]. The µCT images were processed using Avizo 8.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Evaluations were performed using the same samples before cell seeding (day 0) and after 28 days of SHED culture.

Statistical analyses

Data were analyzed using the GraphPad Software (San Diego, USA) through two-way analyses of variances (ANOVA) followed by Tukey's multiple comparison test for DNA, ALP, calcium, osteocalcin, and osteonectin data. Values of $p < 0.05$ were considered statistically significant. These analyses were performed for each group, and experimental time, independently.

Results

SIM release

SIM was slowly and gradually released from scaffolds until the day 90 of the experiment with no latency period (Fig. 1). On day 3 of the experiment, 10% of the equilibrated concentration (M∞) was already released from samples, while on day 14 up to day 28, about 30% to 40% of the M∞ were released. After the 90-day period, the release curve still exhibited an increasing behavior, without reaching an equilibrium (Fig. 1a). The release profile of SIM followed a nonliner sustained-release rate decreasing up to day 21, meaning that up to day 28 it is found the strongest rate release of SIM (Fig. 1b).

DNA quantification

Regarding DNA quantification assay (Fig. 2a), G1 revealed an increase in the DNA content from day 3 to day 21, followed by a decrease to day 28. The groups that were possibly differentiating (G2 and G3) remained mainly stable over the time (no statistical differences from day 3 to day 28), while G4 presented an increase in the DNA content from day 3 to day 28.

Osteogenic differentiation

For ALP activity ($p < 0.0001$, Fig. 2b), extracellular calcium ($p < 0.0001$, Fig. 2c), osteocalcin ($p < 0.0001$, Fig. 2d), and osteonectin ($p < 0.0001$, Fig. 2e), G2 produced notably higher results than the other groups, including the control groups, at all the experimental times. Interestingly, there were an increase on osteocalcin ($p < 0.0001$) and a decrease

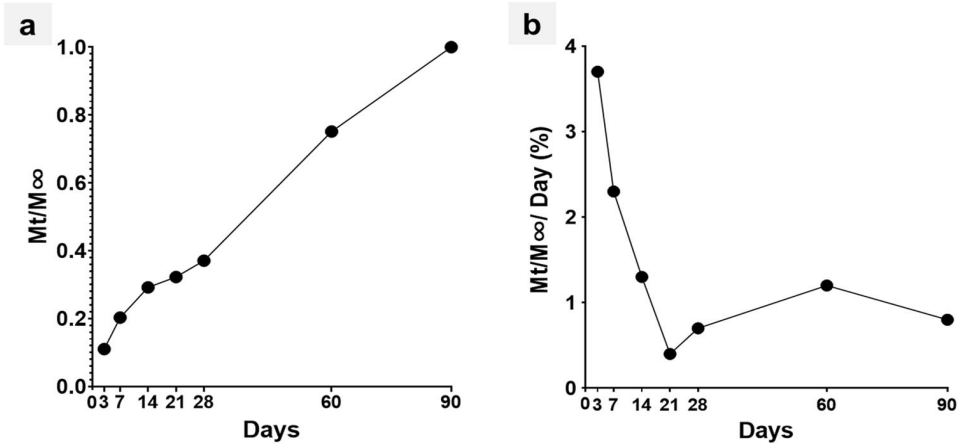


Fig. 1 **a** Simvastatin release from PLGA + HA/βTCP + SIM scaffolds for up to 90 days in phosphate-buffered saline. Calculations were carried out by dividing the absolute amount of simvastatin released at time *t* (M*t*) by the amount of simvastatin at infinity (M∞), which was the equilibrated concentration obtained at the end of the 90-day experimental period. **b** Simvastatin release rate from PLGA + HA/βTCP + SIM scaffolds for up to 90 days. Data was calculated by using the SIM release from the previously measured data (Mt/M∞) expressed as percentage (at $T = 0$ 0% is released and T = 90 100% is released). This was divided by the number of days between each period to show the % of total SIM released per day (%/day)

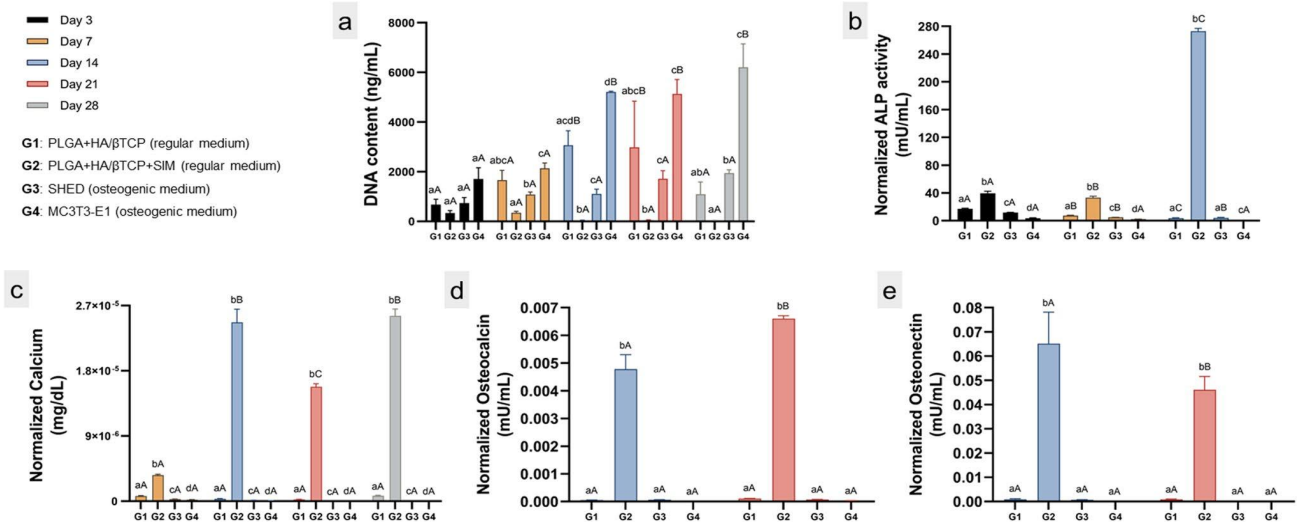


Fig. 2 **a** DNA content assay by PicoGreen® reagent to determine the cell proliferation on days 3, 7, 14, 21, and 28. The DNA content data was used to normalize ALP activity, extracellular free calcium, and osteocalcin and osteonectin proteins. **b** Alkaline phosphatase (ALP) activity on days 3, 7, and 14. **c** Extracellular free calcium on days 7, 14, 21, and 28. **d** ELISA Human Osteocalcin Immunoassay on days 14 and 21, and **e** ELISA Human Osteonectin Immunoassay on days 14 and 21. Different lower-case letters refer to a significant difference (ANOVA/Tukey test, $p < 0.05$) among groups at the same experimental time. Different capital letters indicate significant differences (ANOVA/Tukey test, $p < 0.05$) among the experimental times for the same group

on osteonectin ($p = 0.001$) levels from day 14 to day 21 for G2 (Fig. 2d and e, respectively).

Regarding the mineralization of extracellular matrix (ECM, Fig. 3), G2 and G3 presented high concentrations of mineralized nodules but in a different pattern of deposition. G4 showed less nodules of mineralization than G2 and G3, while G1 showed the least mineralization of the ECM.

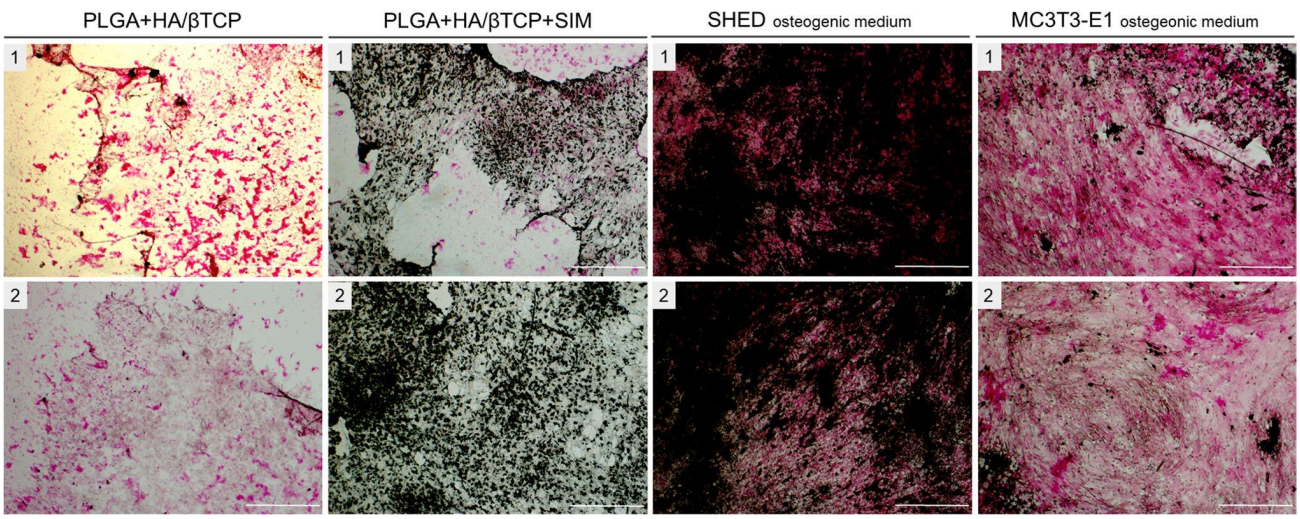
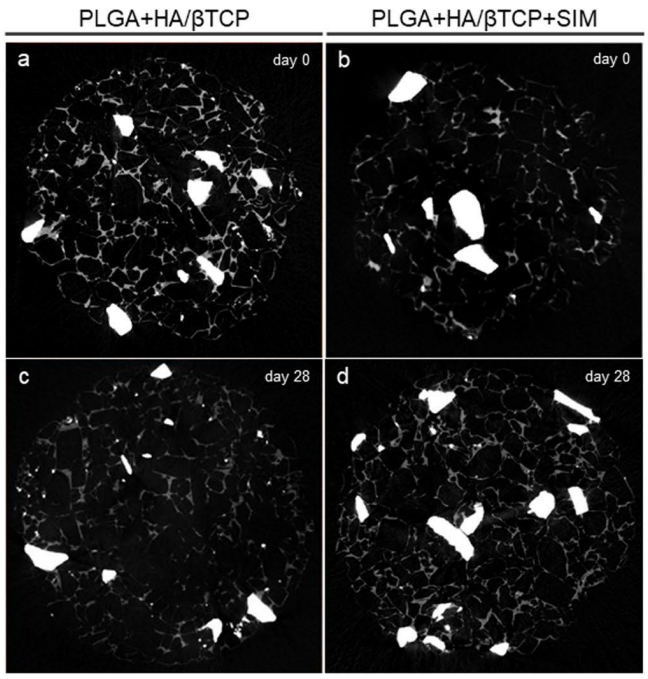
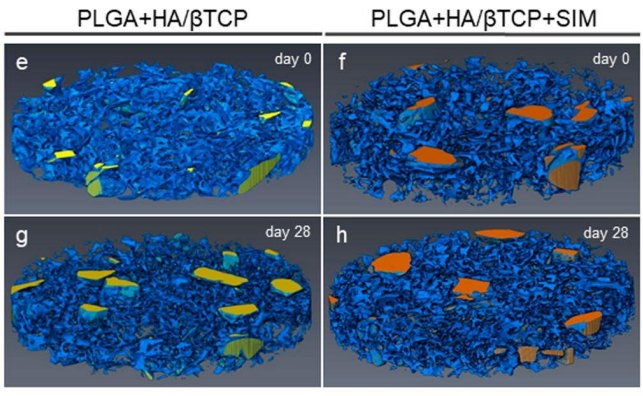


Fig. 3 Von Kossa staining to detect mineralization nodules (colored in brown/black) on day 28. The figure above shows two of the three samples performed. Scale bars represent 500 µm

X-ray microtomography (µCT)

For X-ray microtomography (Fig. 4a-h), gray level 3D images were filtered with "Non-Local Means Denoising" [41] "Unsharp Mask" [42] tools using the AVIZO software and then they were segmented into dense (HA/βTCP), matrix (PLGA), and pore phases. The segmentation process was carried out based on gray level histogram analysis by choosing a threshold that best separates the analyzed phases. After the segmentation, cylindrical volumes of interest (VOI) were outlined for porosity and phase fractions determinations. It can be observed that porosity and matrix phases were similar for both scaffolds and for both experimental times (days 0 and 28); however, the dense phase was more contrasting for PLGA + HA/βTCP + SIM (Fig. 4i). The binary VOIs were modeled into 3D network images. The





i

Groups	Time	Phase fraction (%)		
		Porosity	Matrix	Dense phase
PLGA+HA/βTCP	day 0	87.87	9.62	2.60
	day 28	88.66	7.47	3.87
PLGA+HA/βTCP+SIM	day 0	86.73	7.63	5.65
	day 28	86.40	7.75	5.86

Fig. 4 X-ray microtomography (**a–d**) and 3D network images (**e–h**) obtained before SHED seeding on day 0 and after 28 days of cell culture on the scaffolds. The scaffolds porosity, matrix, and dense phases are presented in the table (**i**)

porosity of about 85 to 90% and the well dispersion of the biphasic ceramic particles across the scaffolds indicate that the samples were successfully produced.

Discussion

Bone regeneration is still a limiting factor in the success of several approaches to oral and maxillofacial surgeries, though tissue engineering using MSC, scaffolds, and osteoinductive mediators might provide a solution [8, 11, 43]. Suitable scaffolds for this purpose continue to be investigated. In our previous research, we developed a biomaterial with adequate structural, chemical, thermal, and biological properties for bone tissue engineering applications [17, 18]. Thus, with the promising results of our aforementioned data, herein we aimed to evaluate the osteogenic capacity of PLGA + HA/βTCP scaffolds embedding SIM in vitro. In summary, we observed that the PLGA + HA/βTCP + SIM group demonstrated results notably higher than the other groups regarding ALP activity, calcium quantification, and osteocalcin and osteonectin proteins quantifications at all the experimental times, even when compared to the positive control groups. Concerning the ECM mineralization analyzed by von Kossa, SHED cultured in osteogenic medium and PLGA + HA/βTCP + SIM group presented high concentrations of ECM mineralization with the nodules deposited in different patterns. Considering the relevance of obtaining a controlled-release system for osteoinduction that has adequate mechanical and chemical properties, degradation rate, biocompatibility, and osteoinduction capacity, these findings are extremely promising for oral and maxillofacial applications. Additionally, the fabrication technique of the proposed delivery system is simple, affordable, and can be mass produced. Also, the raw materials are synthetic and are established in the scientific literature. These factors all contribute to the likelihood that the proposed delivery system could be feasible for use in clinical application [14, 15, 20, 21]. Furthermore, SIM has a good safety profile when applied in appropriate dosages [18, 27, 29, 30, 36, 44]. In addition, additive manufacturing such as bioprinting, using the materials applied herein, could be utilized for the personalized bioprinting of scaffolds for bone defects detected from computed tomography exams.

A deep understanding of the molecular regulatory networks of osteoblast proliferation and differentiation is fundamental to effectively develop biomaterials for bone regeneration [29]. In this context, the methodology of this study was designed with an emphasis on the osteogenic differentiation pathway [44]. The cell commitment to osteogenesis occurs within the first week of osteogenic stimulation when the osteoprogenitor cells enter an early stage of active proliferation followed by a stage of reduction in proliferation due to cell maturation [45, 46]. This initial commitment can be verified by DNA content assay, where increasing DNA is related to more cells, i.e. cell proliferation. Our findings revealed that the groups under strong differentiation stimulus (G2 and G3) remained mainly stable over time.

The early osteogenic markers are present in the first 7 to 14 days of the osteogenic differentiation process and include type 1 collagen and alkaline phosphatase [44, 46]. When preosteoblasts begin their maturation, there is an increase in the alkaline phosphatase activity, which provides phosphate ions for the initial mineralization of the ECM and, consequently, calcium in the extracellular media increases its concentration [44–46]. Such events were proven in the present study by the sharp increase in both alkaline phosphatase activity and concentration of extracellular free calcium on day 14 of the performed analyses, which was more significant in the PLGA + HA/βTCP + SIM scaffolds (G2). Additionally, the release profile of SIM followed a non-liner sustained-release rate. Even at its fastest release rate, in the beginning days, the DNA content and ALP activity results during this period suggested the cells were viable and the SIM released dose was adequate.

Osteocalcin, osteonectin, and osteopontin are considered intermediate proteins in the osteogenic differentiation fate, typically present from days 14 to 21 of cell maturation. Those proteins act on the mineralization of the immature ECM [46]. Herein, the quantifications of osteocalcin and osteonectin validate the alkaline phosphatase and calcium findings, since PLGA + HA/βTCP + SIM scaffolds (G2) promoted the highest protein levels on days 14 and 21 compared to the other groups, including the positive controls. These results are in agreement with other studies that also evaluated SIM playing a role in the osteoinduction of MSCs for bone regeneration [30, 44]. In the late stage of the osteogenic differentiation pathway, mature differentiated cells mineralize the ECM by trapping the free calcium [44, 46]. We have demonstrated by von Kossa staining the presence of mineralized nodules in the ECM of the SHED control group and the group under stimulation of PLGA + HA/βTCP + SIM scaffolds, validating the enhanced osteogenic capacity from embedding SIM [40, 47, 48]. The reduction of calcium on the extracellular media followed by its increase from day 21 to day 28 may be related to the trapping of the free calcium used to mineralize the ECM, which was already mineralized on day 28. The μCT images show that only minor changes in the matrix shape of the scaffolds were observed over a period of 28 days of the experiment, which might be related to the controlled and prolonged release of SIM from the scaffolds, which is favorable for bone regeneration purposes, as already presented in our previous studies [17, 18].

The use of SIM for bone tissue regeneration or in the treatment of other bone diseases was already extensively studied [4–7, 27–29, 36, 44, 48]. SIM seems to act on bone

regeneration through an increase in BMP-2 expression level [25–29, 44]. The primary pathway of osteogenesis includes the BMP-2/Smads signaling pathway, in which BMP-2 triggers the osteogenic signal by phosphorylating Smads, and then mediates the expressions of the genes associated with osteoblast differentiation, such as alkaline phosphatase and osteocalcin [29, 48, 49]. However, the detailed mechanism by which SIM acts on osteogenic differentiation of MSC remains unknown while the signaling pathways that modulate the cell differentiation are considered potential targets of SIM activity [48]. Besides the BMP-2/Smads, Hedgehog and MAPK signaling pathways may be related to the SIM-induced osteogenic differentiation of MSC [44, 48]. Nevertheless, it is important to mention that this is an in vitro study. Further studies on the details of the mechanisms of SIM in the osteogenic differentiation pathway, as well as in vivo and clinical trials, should be performed to confirm our data.

Conclusions

In summary, PLGA + HA/βTCP + SIM scaffolds promoted the highest osteogenic differentiation of SHED. Therefore, the present study revealed the outstanding results of embedding SIM into PLGA and biphasic ceramic scaffolds to differentiate SHED into the osteoblastic lineage. This is mainly due to the development of a biomaterial with adequate structural, chemical, thermal, and biological properties for bone tissue engineering applications, in addition to the successful incorporation method, adequate dosage, and prolonged release of SIM from the scaffolds, which enhanced osteogenic differentiation of dental-derived mesenchymal stem cells.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

Conflict of interest The authors declare no competing interests.

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2.2 ARTICLE 2 - THREE-DIMENSIONAL BIOACTIVE HYDROGEL-BASED SCAFFOLDS FOR BONE REGENERATION IN IMPLANT DENTISTRY

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Review

Three-dimensional bioactive hydrogel-based scaffolds for bone regeneration in implant dentistry

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ABSTRACT

Bone tissue requires a range of complex mechanisms to allow the restoration of its structure and function. Bone healing is a signaling cascade process, involving cells secreting cytokines, growth factors, and pro-inflammatory factors in the defect site that will, subsequently, recruit surrounding stem cells to migrate, proliferate, and differentiate into bone-forming cells. Bioactive functional scaffolds could be applied to improve the bone healing processes where the organism is not able to fully regenerate the lost tissue. However, to be optimal, such scaffolds should act as osteoconductors – supporting bone-forming cells, providing nutrients, and sustaining the arrival of new blood vessels, and act as osteoinducers – slowly releasing signaling molecules that stimulate mesenchymal stem cells to differentiate and deposit mineralized bone matrix. Different compositions and shapes of scaffolds, cutting-edge technologies, application of signaling molecules to promote cell differentiation, and high-quality biomaterials are reaching favorable outcomes towards osteoblastic differentiation of stem cells in *in vitro* and *in vivo* researches for bone regeneration. Hydrogel-based biomaterials are being pointed as promising for bone tissue regeneration; however, despite all the research and high-impact scientific publications, there are still several challenges that prevent the use of hydrogel-based scaffolds for bone regeneration being feasible for their clinical application. Hence, the objective of this review is to consolidate and report, based on the current scientific literature, the approaches for bone tissue regeneration using bioactive hydrogel-based scaffolds, cell-based therapies, and three-dimensional bioprinting to define the key challenges preventing their use in clinical applications.

1. Introduction

Bone regeneration is a subject of major interest in implant dentistry since most of the individuals who need rehabilitation treatments, through implant-supported dental prostheses, have reduced bone thickness and height due to previous dental loss, degenerative diseases, or trauma [1–3]. In addition, for those who still have adequate bone thickness but will undergo dental extractions, strategies to avoid post-surgical bone resorption must be addressed. Despite advances in the clinical application of biomaterials for maxillary bone regeneration, the most predictable methods are still those of filling bone defects where there are still mineralized tissue walls to support bone repair [4,5].

Vertical bone reconstructions are clinically unpredictable and difficult to achieve. Due to its biological and mechanical properties, bone tissue requires intricate strategies to allow the reconstitution of its structure and function. Despite its apparently defined and static structure, bone is a dynamic tissue as a result of the continuous reabsorption by osteoclasts accompanied by the bone remodeling by osteoblasts [4,6–9]. In addition, bone tissue is capable of self-healing and restoring physiological function in minor injuries while major bone defects are still a challenge to repair [1,2,7,10]. Bone healing is a signaling cascade process that includes cell secreting cytokines, growth factors, and pro-inflammatory agents in the defect site that will recruit the surrounding stem cells to migrate, proliferate, and differentiate into functional osteoblasts [7,11].

The clinical application of bioactive scaffolds for vertical bone reconstructions must overcome several challenges before becoming feasible. To be optimal, scaffolds should act as osteoconductors –

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supporting bone-forming cells and sustaining the arrival of new blood vessels that will provide nutrients to the new microenvironment; and should also act as osteoinducers – slowly releasing bioactive molecules that stimulate mesenchymal stem cells (MSCs) to differentiate and deposit mineralized bone matrix [1,2,6,10,12,13]. Besides, the precisely regulated architecture of osteons in the native bone structure indicates that characteristics related to the number, type, and spacing among cells are critical for scaffold design and manufacturing [14,15].

Hydrogels are polymeric networks consisting of crosslinked hydrophilic chains. The high affinity for water provides physical properties such as absorption of large amounts of water or biological fluids while remaining essentially insoluble. Additionally, hydrogels allow exceptional integration with the surrounding tissues, reducing the possibility of inflammatory responses [7,16,17]. Different forms of hydrogels are available from natural to synthetic, increasing their versatility. In addition, their plasticity allows the combined use with other biomaterials to enhance physical properties, the application as a carrier of bioactive molecules, and the use of additive manufacturing techniques, such as the notorious three-dimensional (3D) printing [7,17–21].

There is a high potential for hydrogel-based biomaterials to revolutionize the current clinical approaches to bone tissue engineering. Hence, the objective of this focused review is to consolidate and report, based on the current scientific literature, the approaches for bone tissue engineering using bioactive hydrogel-based scaffolds, cell-based therapies, and 3D bioprinting to define the key challenges preventing their use in clinical applications.

2. Bone tissue engineering

Several approaches have been described to promote bone regeneration resulting from tooth extraction or degradative diseases. Periodontitis is an infectious disease capable to cause irreversible damage in the root cement, periodontal ligament, and alveolar bone, frequently leading to tooth loss [22,23]. Furthermore, after exodontia, substantial bone loss continues to occur due to the lack of function of the alveolar bone and the activity of osteoclasts in the bone walls [5]. Thus, bone loss is a hallmark of periodontitis and the bone defects may vary from small intra-bony defects to large horizontal or vertical defects [22]. Frequently, individuals mutilated by periodontitis demand to rehabilitate the lost tissues since bone loss can cause dimensional limitations for treatments with implant-supported prostheses, and thus it is necessary to resort to regenerative techniques. Despite some promising results with the clinical application of bone grafts – which includes autogenous, allogeneic, xenogeneic, or synthetic biomaterials – associated or not with membranes or barriers (guided bone regeneration), there are still difficulties to reach appropriate vertical bone augmentation [3,4]. Therefore, vertical bone regeneration is normally complex since the grafted site suffers from mechanical compression resulting from mastication or impact generated by conventional prostheses. Furthermore, the management of the soft tissues to maintain a full wound closure and the limited vascularization of the graft can lead to necrosis and graft loss [4,24].

Bone tissue engineering is an interdisciplinary science that applies the principles of biology and engineering to develop biomaterials to substitute, restore, maintain, or improve the functions of the bone [25,26]. The requirements for effective bone tissue engineering are; an adequate number of undifferentiated cells, scaffolds capable of supporting these cells and allowing neovascularization, and signaling molecules able to induce undifferentiated stem/precursor cells to differentiate into bone-forming cells [2,3,6,25,26] *i.e.*, osteogenesis, osteoconduction, and osteoinduction, respectively (Fig. 1).

Several studies have tried to gather the "triad" for bone regeneration, including MSCs, microenvironments, and bioactive molecules, by applying *in vitro* and *in vivo* bioactive protein-conjugated scaffolds [1,2,27–31]. However, none have yet achieved all the requirements to induce *in vivo* bone repair.

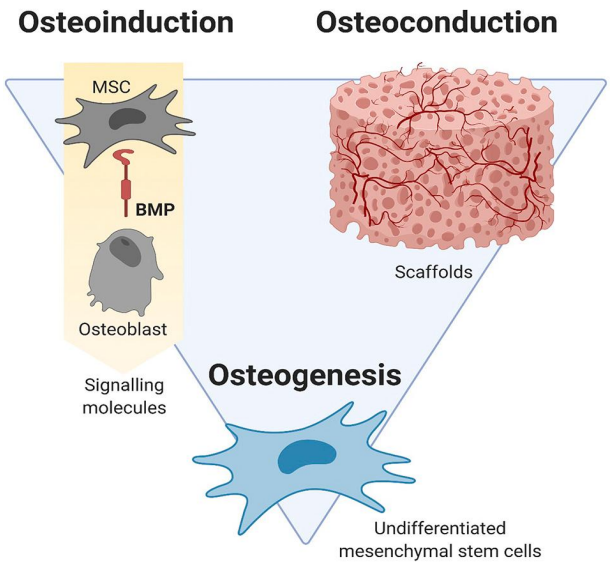

Fig. 1. Schematic representation of the triad of bone tissue regeneration, where osteogenesis is represented by stem cells, osteoconduction is represented by the three-dimensional porous vascularized scaffold, and osteoinduction is represented by signaling molecules able to differentiate stem cells into osteoblasts. The triad must act along to achieve bone tissue regeneration. Created with BioRender.com.

Complex tissues such as bone must be reconstructed to mimic the structural and functional characteristics of the native tissue that has been lost, and this reconstruction must be not only biocompatible but also interactive and integrative with the adjacent tissues. Bone is mainly composed of osteoblasts, osteocytes, osteoclasts, and mineralized extracellular matrix (ECM). Osteoblasts are derived from pluripotent stem cells during embryogenesis or from multipotent bone marrow stromal cells after embryogenesis, while osteoclasts are derived from monocytes. Osteoblasts are active cells able to synthesize and mineralize the ECM. During ECM mineralization, some osteoblasts are trapped in the calcified matrix. Such cells – now called osteocytes, are mature and less active cells [10,32]. Maxillary bones form mostly by intramembranous ossification, different from the bones of the trunk, which usually have endochondral ossification. In contrast to bone regeneration by intramembranous ossification, in which MSCs directly differentiate into osteoblasts, MSCs in endochondral ossification primarily differentiate into chondrocytes to form cartilage which is finally remodeled into bone [33,34]. Intramembranous ossification is characterized by the growth of microcapillary networks into the mesenchymal area, triggering the recruitment and differentiation MSCs into the osteoblast lineage. Osteoblasts secrete and mineralize the ECM around the newly formed blood vessels. As the trabecular bone becomes denser, it coalesces forming grooves around existing blood capillaries. This interconnected immature bone structure is slowly replaced by lamellar bone due to the continuous deposition of bone matrix [34,35]. Thus, when the target is the regeneration of maxillary bones, the most suitable donors are tissues and cells of similar origin.

MSCs represent an attractive route for regenerative medicine applications due to their ability to differentiate into multiple cell lines. The incorporation and delivery of MSCs within a suitable biomaterial helps to maintain the delivered cells at the applied site, supports the integration of the graft with the surrounding tissues, and provides an environment for cells to modulate their osteogenic differentiation [3,29]. These applications require an adequate number of high-quality cells [36,37]. The advantage of using multipotent MSCs is the ease of acquisition since these cells can be obtained from several human oral tissues, such as dental pulp from permanent teeth (DPSC) [1,38], dental

pulp of exfoliated deciduous teeth (SHED) [39], apical papilla (SCAP) [40], dental follicle (DFPC) [41], and periodontal ligament (PDLSC) [42] (Fig. 2), among others. Additionally, MSCs derived from oral tissues have proven to be able to differentiate into bone-forming cells that are appropriate for mineralized tissue regeneration [13,38,39,43].

MSCs are essential for osteogenesis; however, these cells need the support provided by the ECM to properly assume their function. The ECM role might be substituted by the use of scaffolds [6,10], which can be applied as transitory substitutes of the original native tissue that needs to be regenerated. Osteoconductive scaffolds should act to facilitate bone repair while the reconstruction of a bone defect does not reach its complete resolution. Consequently, scaffolds should not act as a permanent substitute for bone tissue [44]. Scaffolds have several requirements: they must be accepted by the organism, provide cell-to-cell interactions, facilitate cell migration and proliferation, enable blood supply, allow biochemical signaling, present degradation rate proportional to bone healing, and keep bone-like stiffness. Therefore, chemical properties, surface topography, internal architecture, porosity and interconnectivity of the pores, stiffness/elasticity, and degradation of its constituents are properties to be adjusted to obtain a functional scaffold [6,12,20,44–49]. Additionally, the architecture of scaffolds can control the quiescence and fate of MSCs [15,31].

In addition to osteogenesis promoted by stem cells and the osteoconduction supported by scaffolds, bone regeneration can be enhanced by the release of signaling molecules. Thus, bone tissue engineering can be enhanced through the delivery of inducing signals in specific microenvironments, allowing controlled differentiation of stem cells into bone-forming cells [31,45,48]. Bioactive factors can be incorporated into scaffolds to promote a gradual release of these drugs, which ideally must be released slowly to exert their action over time without provoking inflammatory reactions. Thus, developing bone substitutes with all the requirements and incorporating signaling molecules that improve bone regeneration is challenging. For this reason, scaffolds that imitate the bone tissue biological features and incorporate bio-signaling molecules are called “smart” scaffolds [1,6,44,48].

3. Mesenchymal stem cells and their applications in bone tissue engineering

Stem cells are directly involved in osteogenesis. MSCs have properties suitable for osteogenesis as they are capable of self-renewal and differentiation. Additionally, MSCs are capable of secreting paracrine mediators and trophic factors, immunomodulation, angiogenesis, and activity in inflammatory reactions [6,7]. Understanding MSCs *in vivo* biology and their relation with local endothelial cells is fundamental for the development of bone regeneration strategies. The correct selection of MSC sources and the preparation of scaffolds with mixed cell formulations might lead to successful tissue regeneration [50].

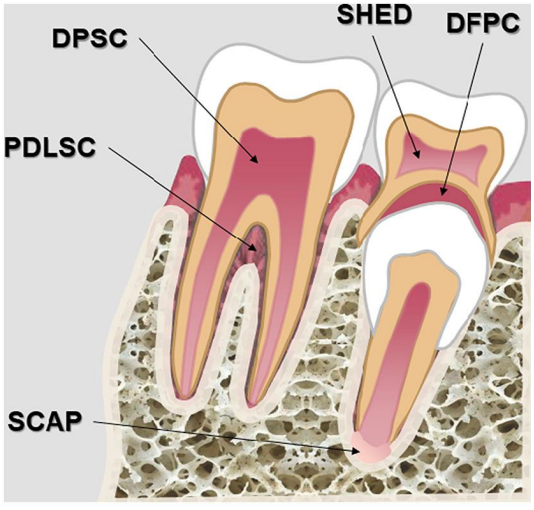

Fig. 2. Schematic representation of some common sources of MSCs in the oral cavity. DFPC: dental follicle progenitor cells; SHED: stem cells from human exfoliated deciduous teeth; DPSC: dental pulp stem cells; PDLSC: periodontal ligament stem cells; SCAP: stem cells from apical papilla.

Adult MSCs are multipotent and typically differentiate into a restricted range of progenitors cells to substitute native tissues. However, the isolation and culture of these cells are facilitated due to the large variability of tissues where they can be extracted. Multipotent MSCs in sufficient numbers have proven to be effective in therapies for bone tissue engineering [6,13,50]. Moreover, MSCs have the ability to both differentiate into osteoblast-like cells, but also produce paracrine factors to initiate the host response to the bone defect site [7].

Oral tissues are a rich source of MSCs and these cells can even be obtained from tissues that would be already removed due to therapeutic purposes. Deciduous teeth, for instance, are an ideal source of MSCs as they are naturally exfoliated. Cell cultures of deciduous tooth pulp results in rapidly expanding cells with *in vitro* properties of MSCs, expressing appropriate markers and capability of differentiation into osteoblasts, chondrocytes, and adipocytes under appropriate stimuli [39]. Another viable option is the buccal fat pad, which is removed for esthetic purposes or to reduce the trauma of the buccal mucosa during mastication [51]. Adipose tissues are rich in MSCs with the capacity of cell differentiation into bone-forming cells *in vitro* [52]. Additionally, other tissues such as permanent dental pulp, dental follicle, and periodontal ligament are frequently removed for therapeutic purposes and their cells might be isolated and cultured since they have already proven to be able to differentiate into different cell lineages [38–42,53]. Table 1 presents oral-derived MSCs, source, and differentiation properties.

There are numerous techniques to extract MSCs from different tissue sources. The processes normally involve isolation and enzymatic digestion of the tissue, incubation of the isolated cells for few days, disposal of non-adherent cells, and continuous culture of adherent cells to the desired passage. Then, a variety of markers are tested for their expression on the cell surfaces to verify cell stemness and some protocols are used for defining multipotent MSCs [59,60]. However, researchers have distinct methods of isolating and culturing MSCs. Different culture conditions, such as medium composition, fetal bovine serum use, supplements, cell seeding density, and oxygen concentration, can affect cell proliferation and differentiation potential [61–63]. The time interval between the tissue extraction from the human body and the tissue processing, and the type and concentration of the enzymes used to promoted tissue digestion can also impair cell viabilities [59]. Also, the cryopreservation method of MSCs can reduce cell viability and differentiation potential, and promote genomic alterations, which further affect MSCs clinical applications [64]. In addition, it should be emphasized that the systemic condition and the age of the donors also affects the proliferation and differentiation potential of MSCs [65].

Another possibility for bone tissue engineering strategies is to focus on the mobilization of resident stem cells. Regeneration mechanisms include the activation, proliferation, migration, and differentiation of resident stem cells, which are recruited to initiate cellular programs leading to tissue regeneration [66–68]. Endogenous MSCs reside within specific tissues and can self-renew and produce defined cell types. Compared with exogenous MSCs, tissue regeneration utilizing endogenous MSCs has reduced cost and avoids surgical morbidity and rejection risks. However, under pathological conditions, such as osteoporosis or periodontitis, the function of resident MSCs is compromised due to the imbalance of tissue components, cell populations, and soluble factors that together regulate the behavior of MSCs [69]. Thus, this imbalance transforms a physiological condition into a pathological condition, leading to reduced abilities of cell proliferation and differentiation, disease aggravation, and limited capacity for tissue healing [69]. Therefore, resident MSCs, as well as their viability, play a key role in the

Table 1
Human dental-derived mesenchymal stem cells and their properties.

Source	Name	Properties	References
Permanent dental pulp	Dental pulp stem cells (DPSC)	Ability to differentiate into osteoblasts, adipocytes, smooth muscle cells, neurons, dentin, dentin-pulp-like complex.	Gronthos et al.; Alge et al.; Saito et al.; Sevari et al. [1,38,43,54,55]
Deciduous dental pulp	Stem cells from human exfoliated deciduous teeth (SHED)	High proliferation rate and cell-population doublings. Expression of neuronal and glial cell markers. Failure to reconstitute dentin-pulp-like complex. Ability to differentiate into osteoblast-like cells and osteogenesis capacity.	Miura et al.; Seo et al.; Aljohani et al. [39,56,57]
Permanent dental apical papilla	Stem cells from apical papilla (SCAP)	Capacity of osteogenic and dentinogenic differentiation, but weak adipogenic potential. Ability to express neurogenic markers.	Sonoyama et al. [40]
Dental follicle from third molar teeth	Dental follicle precursor cells (DFPC)	Ability to differentiate in periodontal ligament-like structures and calcified nodules with bone or cementum like characteristics.	Morsczeck et al. [41]
Periodontal ligament	Periodontal ligament stem cells (PDLSC)	Potential to generate cementum and periodontal ligament-like tissues. They are clonogenic and highly proliferative cells. Adipogenic, osteogenic, myofibroblastic, and neural differentiation properties were identified.	Seo et al.; Coura et al. [42,53]
Buccal fat pad	Buccal fat pad-derived stem cells (BFPSC)	Capacity of osteogenesis.	Bastami et al.; Meshram et al. [48,58]

mobilization of MSCs for tissue regeneration [69].

Several agents targeting pathways, including the Wnt/β-catenin pathway, have been proven to be effective in rescuing compromised endogenous MSCs [69]. Additionally, biomolecules that enhance resident MSCs functions in bone and tooth regeneration comprise growth factors including TGF-β3 and FGF-2 [69], and small molecules such as GSK-3 antagonists [67]. The modulation of the specific microenvironment where MSCs reside *in vivo* is an effective way to regulate endogenous MSCs behavior and, understanding the effects of microenvironmental modulation on resident MSCs, might optimize stem cell-based strategies for bone regeneration [69].

Adult MSCs maintain tissue homeostasis and repair wounds [70]. They reside in specialized niches, which, through intercellular contacts and signaling, influence stem cell behavior. Thus, through the compartmentalization of stem cells into micro-niches, the tissue can control morphogenesis and regeneration [71]. These micro-niches allow the coordination of multi-lineage tissue growth spatially and temporally. The bordering cells provide signals to maintain stem cells in an undifferentiated state; however, once activated, stem cells typically generate short-lived transit-amplifying cells (TACs), which progress to differentiation into lineages that enhance tissue growth [71,72]. Signals generated by neighboring cells can direct if the stem cells will remain quiescent or respond to tissue damage by transforming into TACs to mediate tissue repair [72]. The cell progenitor's ability to choose a lineage is essential in tissue biology; nevertheless, it is unknown when and where stem cells or their progeny become specified. Hence, the spatially and temporally coordinated plan of progenitor heterogeneity implies the importance of progenitor micro-niches in the lineage commitment [71].

Epigenetic analyses are currently pointing to differences among MSCs *in vitro* and *in vivo*. While *in vitro* MSCs are a defined, homogenous cell population following expansion, *in vivo* they are highly programmed according to their resident anatomical tissue location [73]. Different epigenetic landscapes and gene transcription signatures in cells before any *in vitro* expansion evidence this programming [9,73]. Transplantation assays have been the gold standard to analyze self-renewal and tissue regenerative properties of MSCs; however, these methods examine stem cell behavior outside their native context, where they often exhibit broader lineage options than those imposed by their microenvironment [74]. Even though transplantations did not reveal functional distinctions, signs of diversity at transcriptional and epigenetic levels provide persuasive evidence in support of heterogeneity at the stem cell level [74–76]. Additionally, self-renewal and differentiation of MSCs may be affected by epigenetic changes, leading to abnormalities in gene expression. It is also possible that adverse microenvironment provided by bone substitutes may lead to a negative influence in bone regeneration [9]. These concernings lead to additional difficulties in using MSCs and need to be taken into account for bone tissue engineering strategies.

4. Hydrogel-based scaffolds for bone tissue engineering

In the beginning of the 1990s, the concept of scaffold-based tissue engineering was introduced to transpose the low therapeutic efficiency of cells transplanted directly into the grafted area by injection or infusion for cell-based therapies due to the poor retention of cells at the injured site.

There are several limitations regarding the use of tissue-engineered grafts to develop biological substitutes that restore, maintain, or enhance tissue function, such as lack of bone tissue regeneration, insufficient supply of nutrients, and inadequate by-products removal [48,77]. Therefore, it is necessary to provide an adequate microenvironment for cell survival and function, and this might be achieved using 3D engineered biomaterials, *i.e.* scaffolds [6].

For many years, bone autografts were considered the gold-standard treatment for bone regeneration; nevertheless, their therapeutic applications are limited due to the limited availability and, more importantly, the donor-site morbidity [1,29]. Allografts are easy to acquire and manipulate, but may cause immunogenicity [29]. Thus, there is an increasing interest in the development of synthetic bone grafting materials to substitute and augment bone tissue with effectiveness. A variety of synthetic bone substitute materials, including ceramics [7,78–81], metals [20,82,83], and polymers [7,12,27,79–81] have been studied for bone tissue regeneration purposes.

Hydrogels have several potential advantages in bone regeneration. They are polymeric networks consisting of crosslinked hydrophilic chains, which offer mechanical strength and provide nutrient environments suitable for endogenous cell growth. Usually, hydrogels can absorb large amounts of water or biological fluids while remaining essentially insoluble, gradually dissolving over time. The physical integrity of hydrogels in aqueous conditions is guaranteed by physical and/or chemical crosslinking. Likewise, hydrogel mechanical properties can be optimized by crosslinking [20,31,84,85], or by the combination with other structured biomaterials [2,86]. The high affinity for water provides physical properties of living tissue-like hydrogels, such as soft consistency, low interfacial tension with aqueous media and exceptional integration with surrounding tissues, thus avoiding the necessity for surgical removal and reducing the possibility of inflammatory responses [7,16,17].

Injectable forms of hydrogels have been developed as promising biomaterials for tissue engineering applications as they are generally biocompatible, biodegradable and can mimic ECM architecture while they are less invasive and easy to apply clinically. With the progress of cellular therapies, there is an increasing need to develop injectable hydrogels to carry cells, preventing the need to perform open surgery and allowing minimally invasive approaches for the application of biomaterial and cells [1,20,28,81,87].

Hydrogels can be designed from a wide variety of materials, from totally synthetic to natural, which might be used in different combinations. Furthermore, they can be custom-made to obtain the desired geometry for implantation or injection, while the degradation rate, architecture, stiffness, porosity, and drug release profile can be controlled by the crosslinking method [7,17–21].

Natural hydrogels are proteins or polysaccharides derived from biological sources, such as collagen, gelatin, albumin, fibrin, silk proteins, matrigel, glucan, hyaluronic acid, chitosan, agarose, bacterial cellulose, and alginate [7,18,21,82]. Several of the natural hydrogels are obtainable through the extraction from their natural source. For example, collagen, fibrin, and hyaluronic acid are natural constituents of the ECM, while alginate and agarose are derived from marine algae [18]. Natural hydrogels have adequate biocompatibility and low immune response. They allow cell adhesion, proliferation, and new tissue regeneration, and are absorbed through metabolic or enzyme-controlled degradation [7]. Nevertheless, like any natural material, there are disadvantages concerning variations in the quality of the material, culminating in low reproducibility and limited control of the mechanical properties [1,2,16,18,82].

Synthetic hydrogels include polymers, such as poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), poly (hydroxyethyl methacrylate) (PHEMA), poly(methyl methacrylate) (PMMA), poly(acrylamide) (PAAm), cellulose [7,16,82]. Synthetic hydrogels have a defined structure, reliable material sources, long shelf lives, reduced risk of immunogenicity, and may be manufactured in large quantities with reproducibility. The main limitations are their poor bioactivity, acid by-products, and highly repetitive structure, which offers no biological information to cells [7,18,88]. Therefore, synthetic hydrogels might be used in conjunction with biological materials to compensate those disadvantages [7]. As a review, Table 2 presents some of the latest hydrogel-based scaffolds used for osteogenic regeneration purposes.

With the advent of rapid prototyping, soft lithography, photopatterning, electrospinning, and additive manufacture techniques, such as the 3D bioprinting, it has become possible to construct systems with hierarchical architecture composed of hydrogels and cells, as well as microfluidic channels for vessel permeation, mimicking the vascularization of complex tissues or organs [7,18,34,89–91]. Thus, these technologies benefit the development of research in basic biology, molecular signaling, and tissue engineering [18,36].

Among the countless scaffolding techniques designed to regenerate bone tissue, 3D printing is a particularly promising technology [2,10]. 3D printing employs automated manufacturing processes, enabling the creation of scaffolds by managing pore size, porosity, and interconnectivity as required [2,99]. Hence, scaffolds produced with interconnected pores must allow the formation of vascularized tissue, which is necessary to provide nutrients and oxygen to the proliferating and differentiating cells [27,34]. Moreover, 3D printing techniques could be applied in conjunction with imaging techniques such as computed tomography and magnetic resonance to generate patient-specific 3D tissue models, allowing the fabrication of customized structures for areas of bone defects. Computer-aided design (CAD) of models of the defect could be used to develop a personalized treatment, enabling to print scaffolds that replicate patient-specific anatomy [2,20,100,101] (Fig. 3).

Thus, the benefits of 3D printing include the ability to control the internal and external 3D architecture of scaffolds, the production of scaffolds that accurately match patient-specific requirements, the manufacturing of scaffolds with multiple biomaterials, and the predictable mechanical response by pre-defining scaffolds architecture [101,102]. Nevertheless, it is important to differentiate the terms: the term 3D printing is often used to describe the fabrication of inert or bioactive scaffold materials without the presence of living cells, whereas 3D bioprinting usually denotes to the printing of cells and scaffolds together or dense aggregates of cells free from scaffold support [101,102].

The maintenance of bone grafts after implantation is dependent on how fast they can vascularize. Merging 3D bioprinting of scaffolds with strategies for prevascularization might guarantee the establishment of a vascular supply throughout the graft and the suitable morphology of the forming bone [12,14,34]. Since the lack of vascularization may constrain osteogenesis and host integration, inhibiting the repair of large defects, *in vitro* and *in vivo* strategies have been explored to generate vascularized bone grafts, including biochemical stimulation or co-culture of endothelial cells with MSCs [12,14,29,103,104]. Co-culture of human umbilical vein endothelial cells (HUVECs) and MSCs is one of the most applied options since it can create a vascular network while regenerating the target tissue [14]. In addition, HUVECs are known to secrete growth factors such as insulin growth factor-1 (IGF-1), endothelin-1, and BMP-2, enabling the promotion of osteogenic differentiation of MSCs [14,105,106]. Thus, scaffolds with co-culture of HUVECs and MSCs could support suitable neovascularization and, consequently, enable osteogenic differentiation of MSCs and bone regeneration [14,15]. Nevertheless, it is important to highlight that HUVECs are isolated from veins of umbilical cords and, as other human tissues manipulation, should be obtained with informed consent and local ethics approval [107].

Apatite crystals strengthen the collagen matrix of biomineralized tissues such as bone and teeth [108]. Hydroxyapatite is the main inorganic constituent of bone, corresponding to 60% of the bone matrix [87]. Thus, calcium phosphates are the first choice for synthetic bone substitutes since they are similar to the bone matrix, osteoconductive, and still provide mechanical resistance to the bone substitutes [2,7,82,99]. Hydrogels loaded with calcium phosphates have been produced to improve both mechanical properties and bioactivity of hydrogel-based scaffolds. The addition of hydroxyapatite in hydrogels has enhanced cell viability and proliferation, as well as osteogenic differentiation of MSCs [1,29,87], and stabilize the network of hydrogels improving the resistance to mechanical loads, such as compressive strength [20,21,48,81].

Similarly, bioactive glasses (BGs) have been shown to be excellent biomaterials due to their high biocompatibility and bioactivity, angiogenic effect, and antibacterial activity induced by ion release. BGs are a group of surface reactive glass-ceramic biomaterials developed by Hench in 1969 that are composed of SiO_2, CaO, Na_2O, and P_2O_5 at different proportions [109,110]. They are able to induce osteogenic differentiation of stem cells by releasing biologically active ions such as Ca^{2+}, Mg^{2+}, and PO_4^{3-} [111–113]. BGs bind to bone by forming a layer of carbonated hydroxyapatite, which mimics the mineral phase of bone, therefore resulting in a biological combination between BGs and bone tissue. Additionally, BGs have an amorphous nature that allows the delivery of biomolecules at a sustained rate [113]. However, the role of BGs for regenerative medicine should be completed with the application of 3D scaffolds development. As such, the current interest in BGs focuses on their ability to stimulate the growth of new bone and their potential to be applied together with biomaterials to improve the bone regeneration mechanisms [110]. Thus, the incorporation of BGs into hydrogel 3D structures not only makes the mechanical properties of the scaffold more similar to the host bone but also participates in the bioactivity of the material [1,82,112,114,115].

Table 2
Hydrogel-based scaffolds for bone tissue engineering, their applications and outcomes.

Material	Type/source	Application	Outcomes	References
Alginate-Matrigel hydrogel loaded with bioactive glass microparticles	Natural Alginate was derived from sea algae; Matrigel was derived from basement membrane proteins extracted from mouse tumor	*In vitro* (human dental pulp MSCs)	The presence of Matrigel in the hydrogel composite increases the osteogenic differentiation of MSCs, despite the decrease in elasticity of the hydrogel with the addition of bioactive glass microparticles.	Sevari et al. [1]
3D-printed heparin-conjugated collagen matrix encapsulating MSCs, reinforced with β-TCP nanoparticles scaffold and functionalized with recombinant human bone morphogenetic protein type 2 (rhBMP-2)	Natural Collagen is the main component of natural bone extracellular matrix	*In vitro* (human dental pulp MSCs); *In vivo* (rat dorsum defects)	*In vitro*: capacity of heparin-conjugated collagen matrix to maintain the bioactivity of rhBMP-2 and support MSCs viability and osteogenic differentiation. *In vivo*: capacity of osteogenic differentiation of MSCs and induction of ectopic bone formation.	Fahimipour et al. [2]
Bacterial cellulose loaded with bone morphogenetic protein type 2 (BMP-2)	Natural Bacterial cellulose gel was obtained by culturing *Acetobacter hansenii*	*In vivo* (frontal sinus lift rabbit model)	Bacterial cellulose presented excellent biocompatibility. When combined with BMP-2, bacterial cellulose allowed bone regeneration and served as both barrier membrane and sustained-release drug carrier proved through histological and immunohistochemical evaluations.	Koike et al. [92]
Chitosan-Gelatin hydrogel incorporating nanodimensional bioactive glass particles	Natural Chitosan was derived from shrimp shells; Gelatin was derived from bovine skin	*In vitro* (human dental pulp MSCs); *In vivo* (rat femur defects)	*In vitro*: Cytocompatibility and ability to induce the crystallization of bone-like apatite; *In vivo*: Chitosan-Gelatin hydrogel incorporating 5% of nanoparticles of bioactive glass significantly produced the greatest amount of new bone among the tested groups.	Covarrubias et al. [93]
Composite bisphosphonate-linked hyaluronic acid-calcium phosphate hydrogel	Natural Hyaluronic acid is a polysaccharide widely distributed in the ECM of eye's vitreous humor, synovial fluid, skin, and umbilical cord	*In vivo* (sinus lift rabbit model)	Synthetic granular calcium phosphate compound and deproteinized bovine mineral graft induced more bone regeneration than hyaluronic acid-calcium phosphate hydrogel at a histomorphometric evaluation.	Trbakovic et al. [94]
Gelatin-coated β-tricalcium phosphate (βTCP) scaffolds with rhBMP-2-loaded chitosan nanoparticles delivery system	Natural Chitosan was derived from marine crustaceans; Gelatin was derived from porcine skin	*In vitro* (human buccal fat pad MSCs)	Gelatin-coated βTCP scaffolds with rhBMP-2-loaded chitosan nanoparticles were able to support cell viability and attachment and slowly release of rhBMP-2 in a therapeutic dose that permitted osteogenic differentiation of MSCs.	Bastami et al. [48]
Alginate-gelatin methacrylate (GelMA) hydrogel	Natural Alginate was derived from brown sea algae; GelMA was derived from denatured collagen	*In vitro* (human gingival MSCs and human bone marrow MSCs)	The addition of GelMA to alginate jeopardizes the hydrogel osteogenic differentiation induction of encapsulated MSCs, which is attributed to the decrease in the elasticity of the hydrogel. The osteogenic differentiation capacity of MSCs is regulated by the alginate-GelMA physiochemical properties and the presence of inductive signals.	Ansari et al. [31]
Crosslinked pNIPAM-co-DMAc hydrogel loaded with hydroxyapatite nanoparticles	Synthetic, injectable	*In vitro* (commercial human MSCs); *In vivo* (rat femur defects)	*In vitro*: capacity to induce osteogenic differentiation of commercial human MSCs; *In vivo*: biocompatibility, ability of integration with surrounding tissues and increased deposition of bone regeneration early markers.	*In vitro*: Thorpe et al. [30] *In vivo*: Thorpe et al. [29]
3D-bioprinted biphasic osteon-like scaffold, containing hMSCs and human umbilical vein endothelial cells (HUVECs) encapsulated in a fibrin-polycaprolactone hydrogel	Semi-synthetic; Fibrin was prepared by mixing fibrinogen from bovine plasma and type A porcine gelatin Polycaprolactone is a biodegradable polyester	*In vitro* (commercial hMSCs and HUVECs) *In vivo* (rat cranial bone defects)	*In vitro*: significant increase in gene expression of angiogenic markers. *In vivo*: histological analysis of explanted scaffolds showed a significant increase in the number of blood vessels per area (capacity to improve the neovascularization) in the bioprinted osteon-like scaffolds.	Piard et al. [15]
Sodium alginate/hydroxyethylcellulose/ hydroxyapatite composite	Semi-synthetic	*In vitro* (commercial human MSCs); *In vivo* (rat femur defects)	*In vitro*: cell viability of hMSC and proliferation capacity of the tested hydrogel composite scaffolds. *In vivo*: histological examination showed formation of new bone to repair the defected sites after 6 weeks of scaffolds implantation.	*In vitro*: Tohamy et al. [95] *In vivo*: Tohamy et al. [96]
3D polyvinyl alcohol-tetraethylorthosilicate-alginate-calcium oxide biocomposite cryogels	Semi-synthetic	*In vivo* (rat cranial bone defects)	Promotion of regeneration of the bone defect while simultaneously resorption of its contents from the defect site over the period of 4 weeks. Osteoblastic activity at the defect site with an increase of the differentiation towards osteoblastic lineage and maturation of osteoblasts from 2 to 4 weeks.	Mishra et al. [97]
Triblock poly(ethylene glycol)-poly (ε-caprolactone)-poly(ethylene glycol) copolymer, collagen and nano-hydroxyapatite	Semi-synthetic, injectable	*In vivo* (rabbit calvarial bone defects)	Evaluation of bone regeneration was performed for 4, 12, and 20 weeks. Radiological and histological analyses revealed new bone tissue formed from the edge of defects and surface of native bone towards the center. Great potential to repair the non-loading defects with a minimally invasive surgery.	Fu et al. [98]

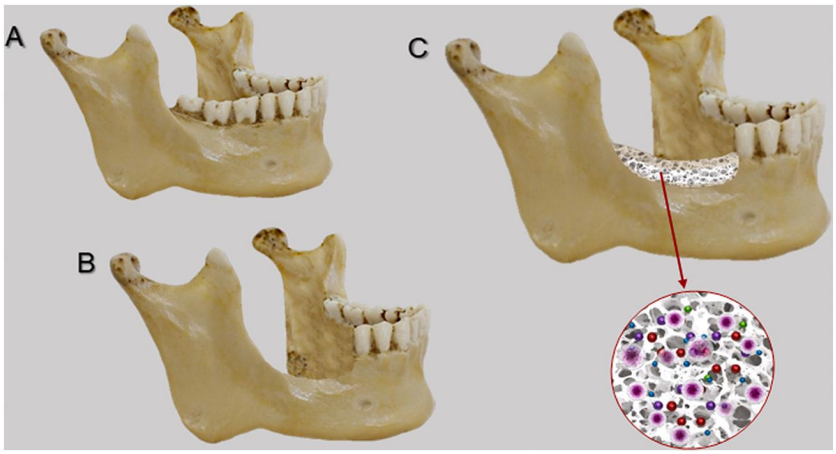

Fig. 3. Schematic representation of dental loss resulting in bone remodeling/resorption with consequent reduction of anatomical dimensions. A) Normal mandibular anatomy with complete dentition. B) Tooth loss leads to bone resorption, with loss of function and limited bone dimensions for adequate implant-supported rehabilitation. C) Three-dimensional image exams might generate models to allow the manufacturing of customized bone grafts with different biomaterials and compositions.

5. The application of hydrogels in three-dimensional cell cultures

The use of human MSCs in biomedical research requires the development of efficient and cost-effective systems for cell isolation, proliferation, and differentiation [116]. In general, stem cells require biological signals from their substrate to promote cell survival and rapid proliferation, which must be provided by culture systems [36]. Two-dimensional (2D)-based cell culture systems suffer from inherent heterogeneity and limited scalability and reproducibility. An attractive approach for scaling up production is to move cell culture from 2D to 3D systems [36,117,118].

In recent years, it has become clear that conventional models of 2D cell cultures for basic research, such as cytotoxicity and proliferation assays, drug screening, and tissue engineering have limitations. In 2D cultivation systems, the growing cell attaches to one side of plastic while the other side of the cell remains exposed to the culture medium. Thus, cell-to-cell interactions in the monolayer are established merely at the cellular periphery and this cultivation condition is different from *in vivo* situations (Fig. 4) [18]. Thus, in contrast to traditional 2D cell cultures, 3D cultures provide architectural and material diversity to understand the cellular microenvironments and guide the comprehension of the basic components of biological systems that are integrated into the dynamic tissue physiology [19,46,49].

Cell-to-cell communication and interactions of cells with the ECM plays a key role in the perception and reaction to external stimuli [119]. Substantial changes in gene expression profiles in various cell lines of 3D-compared to 2D cell culture conditions have been reported in the literature [120–124]. That is, there is a strong impact on the way cells behave in face of the molecules or biomaterials for regenerative medicine. Alternatively, 3D culture conditions support self-renewal and promote stemness maintenance of embryonic stem cells compared to 2D cultures [125].

There are several methods to develop 3D cell cultures, such as cell aggregates (carrier-free microspheres where cells are enveloped by others), synthetic scaffolds (*e.g.* polysterol), naturally derived matrices (*e.g.* ECM derived from decellularized tissues), and hydrogels. Particularly, hydrogels are an innovative approach for 3D cell cultures, providing numerous arrangements of synthetic and natural molecules to mimic *in vivo* tissue architecture [18].

To achieve efficient stem cell expansion at high densities, hydrogel-based 3D systems must overcome engineering challenges and support certain biological aspects of each cell line in particular [36,37]. A fundamental property of hydrogels in 3D cultivation is the ability to provide appropriate transport of nutrients, oxygen, protein factors, and residues throughout their structure. Mass transport inside the hydrogels occurs by diffusion, which is affected by the nanoporosity of the biomaterial [18,36,37,126]. With the advent of 3D bioprinting, it has become possible to construct systems with hierarchical architecture composed of hydrogels and cells, as well as microfluidic channels for vessel permeation, mimicking the vascularization of complex tissues or organs [7,18,34,89–91]. Thus, this technology benefits the development of research in basic biology, molecular signaling, and tissue engineering. However, they remain at their early stages of development and have issues specifically relating to the mechanical stability of microfluidic channels and poor perfusion of networks [18,36].

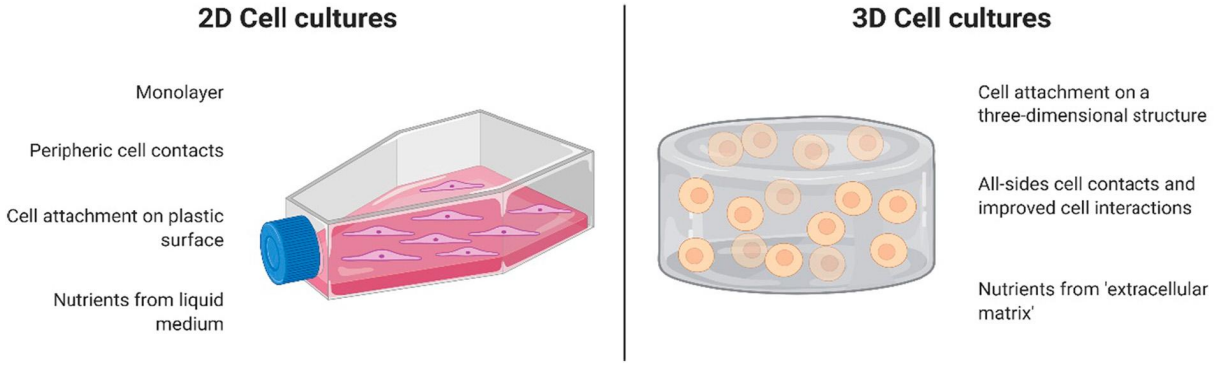

Fig. 4. Comparison between two- and three-dimensional cell cultures. Three-dimensional cultures offer several advantages, mainly due to the improved cell-to-cell interaction provided by the extracellular matrix (ECM), which supports cells and delivers nutrients, providing a structure that better resemble the *in vivo* condition. Created with BioRender.com.

Hydrogels are undoubtedly beneficial 3D systems for different applications in cell cultures. These systems aim not only at the deep understanding of basic cellular processes but also improve tumor models, bring new methods for drug screening and cytotoxicity, as well as develop cell-based therapy and tissue engineering [1,18,49].

6. Bioactive hydrogel-based scaffolds for bone tissue engineering

Hydrogels are commonly used as scaffolds in tissue regeneration since they are excellent environments for cells and, additionally, can incorporate signaling molecules to improve the regenerative capacity through the controlled release of these molecules [2,21,29,48,116]. Due to the hydrogel network structures, embedded signaling molecules are confined in the crosslinked meshes, which enables to control the release of the molecules as desired [7].

Stimulus-responsive hydrogels are of particular interest for use in craniofacial tissue engineering as they may respond to environmental changes to alter network structure, swelling behavior, permeability, or mechanical strength, as well as control the release of biosignaling drugs [7,31,127]. Several physical and chemical stimuli have been applied to "smart" hydrogel systems. Physical stimuli include temperature, electric field, light, and solvent composition. However, chemical and biochemical stimuli such as pH, ionic strength, and molecular recognition are most commonly explored for oral delivery [2,7,17].

The controlled release of signaling drugs have been reported to significantly enhance the regeneration capacity of inert biomaterials. Growth factors are naturally occurring proteins that are an integral component in controlling cellular functions, such as migration and proliferation, and determining cell fate [7,21,48]. Although significant osteogenic potential has been evidenced by growth factors, such as the bone morphogenetic protein (BMP) and platelet-derived growth factors, their clinical application is limited due to variations of their physical properties during the incorporation procedure, short half-life, immunogenicity complications, rapid distribution by body fluids, large doses required, inactivation by inflammatory cytokines, inefficient tissue formation, and clinical side effects [2,8,25,48,128,129].

Since the majority of signaling molecules are proteins, direct delivery may result in enzymatic degradation in the ECM. In addition, the delivered biomolecules may spread to surrounding tissues, leading to adverse effects, such as inflammatory responses [7]. Thus, the loading method of the signaling drugs into the hydrogel-based scaffold determines the tissue regenerative outcomes. It is fundamental that the hydrogel maintains the biological activity of the delivered constituents to provide sustained effective concentrations at the treatment site with a controlled release rate [7,48].

Ideally, bioactive signaling molecules should be gradually released at the applied site in concentrations that do not interfere with cell viability [130]. The most common loading procedures include physical mixing, which has inadequate release control. Hence, alternative incorporation approaches of bioactive agents into hydrogels are suggested. Chemical conjugation favors the concentration of the bioactive molecules. However, as stated above, conjugation may alter the protein stability during the incorporation procedure [131], while synthetic peptides can be modified to contain functional chemical groups that favor hydrogel crosslinking without altering the stability [116]. Peptides are small molecules compared to proteins and due to their smaller size, they can be easily synthesized, metabolically cleaved, rapidly eliminated from the organism, avoiding accumulation in specific organs and minimizing toxic side effects [116,132]. Hence, synthetic peptides have been proposed as an alternative to the use of growth factors since they are sufficiently stable, less immunogenic, and their incorporation into biomaterials is facilitated once they are not so sensitive to changes on temperature and pH [133,134]. The most commonly applied peptides are the ones of cell adhesion peptides containing arginine-glycine-aspartic acid (RGD) sequence, which is present in collagen I, fibronectin, bone sialoprotein, and osteopontin. These peptides are promoters of osteogenic differentiation [135].

Similarly, small molecules have been proven to have osteoinductive potential. Small molecules have physical properties that can minimize the limitations of growth factors since they are normally more stable, highly soluble, and low-cost. However, like every signaling mediator, small molecules can have side effects, including toxicity at high dosage or potential damage to surrounding tissues [136,137]. Small molecules may act as co-activators of BMP-2 [136] or activators of Wnt/β-catenin pathway as a biomedical strategy [66,136–138]. The activation of the Wnt/β-catenin pathway is an early response to tissue damage, which stimulates cellular-based tissue repair [66]. The key signal intermediate in Wnt signaling is β-catenin, which is degraded in the cytoplasm by a multiprotein destruction complex consisted of Axin, tumor suppressor *adenomatous polyposis coli* gene product (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1). This complex traps and phosphorylates β-catenin, targeting its ubiquitinoylation and proteasomal degradation [139]. With reduced β-catenin levels, Wnt signaling pathway is inactive, compromising tissue renewal, and triggering diseases such as osteoporosis [138]. Thus, inhibitors of GSK3β are applied as therapeutic candidates to promote bone regeneration [66,138]. Nevertheless, despite the promising results regarding GSK3β inhibitors, it is important to bear in mind that GSK3β is implicated in many other critical cellular functions, which includes cell cycle control, proliferation, differentiation, and apoptosis [140].

A list of signaling molecules, including the main growth factors, involved in the pathway of osteogenic differentiation is presented in Table 3, while the schematic representation of osteogenic differentiation pathway of MSCs is represented in Fig. 5.

Local delivery of therapeutic agents, particularly synthetic peptide-based drugs, is of great interest for the safe and controlled administration of drugs in the clinical routine. Hydrogels are an outstanding option for oral drug delivery because of the adaptive parameters that allow the controlled release of various therapeutic and bioactive signaling molecules. Nevertheless, further studies are required to more accurately simulate physiological conditions and enhance hydrogel performance, which is essential to achieve bioavailability, biocompatibility, and bioactivity. The main purposes in the development of drug delivery systems are to protect bioactive therapeutic molecules from early degradation, enhance drug efficacy, and minimize toxic side effects. Ideally, controlled release systems should maintain the drug concentration within a therapeutic window over an extended period [17,146].

7. Discussion

Bone is an organ capable of regeneration; however, some clinical situations require enhancement of bone repair to ensure the rapid restoration of physiological functions [26]. Although hydrogels and their compositions used as scaffolds present several advantages for bone tissue regeneration, some issues are still to be solved. The first disadvantage of hydrogels is their lower mechanical strength compared to native bone. However, several recent studies have resolved this issue by reinforcing the hydrogel crosslink nets or by combining different biomaterials [2,20,31,84–86], especially calcium phosphates, which give the cells additional information on the type of tissue they should differentiate. In this regard, bioactive glasses are promising since they are highly related to bone regeneration, in opposition to hydroxyapatite, for example, which is the main component of dental hard tissues too [1,111,112,114,115,147]. Second is the vascularization of the scaffold after implantation, which is essential for biomaterial integration and cell nutrition. Osteoblasts in healthy bone tissue states exist in high oxygen conditions, in contrast to what is seen in cartilage. The cellular response to the spatial variations in oxygen condition between bone and cartilage is mediated by the hypoxia-inducible factor (HIF). HIF plays a key role in regulating both chondrogenesis and osteogenesis by guiding cell differentiation, promoting appropriate ECM production, and maintaining

Table 3
Signaling molecules involved in the osteogenic differentiation pathway.

Growth factor	Outcomes	References
BMP-2: Bone morphogenetic protein type 2	Effect on differentiation of MSCs in osteoblast precursor cells and osteoblast like cells. Able to induce bone morphogenesis. It is the most studied and clinically applied molecule for bone regeneration purposes. It plays an important role in differentiation of MSCs, regulating important genes such as alkaline phosphatase, osteocalcin, collagen type-1, and bone sialoprotein. BMP-2 can also trigger the activation of mitogen-activated protein kinase (MAPK) pathway, which acts on cell commitment and differentiation in osteoblastic lineage.	Wildemann et al.; Rahman et al.; Bilem et al.; Venkatesan et al.; Shakya & Kandalam [8,20,21,135,141]
BMP-4: Bone morphogenetic protein type 4	Involved in bone induction, fracture repair and tooth development.	Rahman et al.; Venkatesan et al. [8,21]
BMP-7: Bone morphogenetic protein type 7	Potential osteoinductive factor for epithelial osteogenesis. Role in bone homeostasis and calcium regulation.	Rahman et al.; Venkatesan et al. [8,21]
TGF-β1: Tumoral growth factor β	Act along with BMPs to modulate MSCs differentiation during bone development, formation, and homeostasis.	Thrivikraman et al. [102]
IGF: Insulin-like growth factors	Mediates the effects of hormone, growth factors, cytokines, and morphogens during fracture healing process. Strongly stimulates proliferation and chemotaxis of cell populations, playing important role in bone metabolism.	Lee & Shin; Thrivikraman et al. [102,142]
VEGF: Vascular endothelial growth factor	Strongly expressed during bone fracture repair. It is necessary for bone healing, but it is not osteoinductive. Enhances local angiogenesis. Stimulates proliferation and migration of endothelial cells. Acts on the recruitment, survival, and activity of bone forming cells. Beneficial in the treatment of critical bone defects by promoting the differentiation of osteoprogenitor cells and mineralization of the repaired bone.	Lee & Shin, Keramaris et al., Calori et al., Shakya & Kandalam [20,142–144]
FGF: Fibroblast growth factor	Strongly expressed during bone fracture repair. It is necessary for bone healing, but it is not osteoinductive. Presents angiogenic effect.	Calori et al. [144]
PDGF: Platelet-derived growth factor	Presents chemotaxis effect over MSCs and enhancement of bone formation. However, its function in bone regeneration is controversial among literature.	Hosseinpour et al.; Shakya & Kandalam [20,145]
Shh: Sonic hedgehog	It is involved in fracture healing and bone maintenance. It has its effects related to Gli2, a transcriptor factor able to activate BMP2 gene expression. Shh-induced osteoblastogenesis occurs through Runx2.	Rahman et al. [8]
Runx2: Runt-related transcription factor 2	It is a transcriptional regulator of osteogenic differentiation and bone formation. It is a key osteoblast fate specifying transcription factor. It is modulated by FGF. It is a well-known early osteogenic marker.	Bilem et al.; Rahman et al. [8,135]
βcatenin/Wnt	It is involved in several steps of osteogenic differentiation process. As soon as MSCs become committed to osteogenic lineage, the activation of βcatenin/Wnt signaling enhances the bone formation through the renewing of stem cells, stimulation of preosteoblast proliferation, induction of osteoblastogenesis, and inhibition osteoblast apoptosis.	Bilem et al.; Rahman et al. [8,135]
miRNA or MiR: Micro RNA	They can regulate the expression of genes during osteogenic differentiation pathway of MSCs, leading to bone formation. MiR-208, for example, is interconnected with BMP-2 and Runx2 on the enhancement of bone regeneration. However, some miRs may act as negative regulators of osteogenic differentiation of MSCs.	Rahman et al. [8]

of the correct cell phenotype [148]. Thus, scaffolds should be highly hydrophilic, porous, and reduced as possible in size to allow vascularization and oxygen nutrition, which could be controlled during the personalization of 3D models, as shown in Fig. 3. In addition, the co-culture of vascular cells and MSCs has been proposed for 3D bioprinting with encouraging results in *in vitro* and *in vivo* studies [14,15,105,106].

It is imperative to bear in mind that intramembranous ossification produces most of the craniofacial bones while endochondral ossification predominates on the long bones of the trunk [33,34]. Intramembranous ossification is characterized by the growth of microcapillary networks into the mesenchymal area, triggering the recruitment and differentiation MSCs into an osteoblast-like lineage [34,35]. Furthermore, the majority of craniofacial bones are generated from the neural crest and do not express Hox genes, while the caudal skeletal structures and mesodermal-derived osteoblasts express Hox during development [149]. Thus, the use of trunk skeletal models, such as rat dorsum or femoral bone defects models [2,29,93,96], are normally inappropriate for craniofacial bone tissue engineering, the target for implant dentistry, while maxillary sinus lift models seem to be a more adequate strategy [92,94].

The regulated architecture of osteons in bone structure indicates that features related to the number, types, and spacing among cells might be critical to tissue function [14,15], but this is normally not taken into account when producing hydrogel-based scaffolds while the 3D bioprinting technology could be used on its favor to benefit this shortcoming. Nevertheless, bone is a dynamic tissue [4,6–8], and for its regeneration, interconnected signaling will be responsible for final target gene expressions required for osteogenesis [8] and this is a

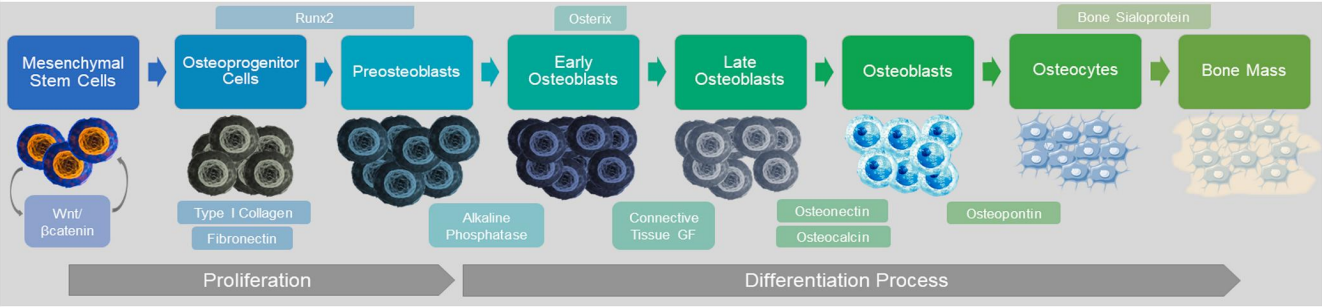

Fig. 5. Schematic representation of osteogenic differentiation pathway that mesenchymal stem cells experience and the proteins, growth factors, and molecules involved in the process. The image represents the sequential stages of the osteoblastic differentiation process, starting with the decrease in cell proliferation rates and the commitment of progenitor cells to preosteoblasts. Successively, preosteoblasts differentiate into mature osteoblasts able to deposit extracellular bone matrix, followed by differentiation into osteocytes and mineralization of the matrix.
Adapted from Rahman et al. [8].

difficult process to replicate.

Most tissue engineering approaches require seeding of high densities of cultured MSCs over scaffolds to mimic the cellularity of native tissues and create sufficient cell-secreted ECM to form a functional graft. However, it was recently shown that high-density 3D culture in hydrogel scaffolds stimulated MSCs to remain quiescent while low-density culture favored the active phase of cell cycle and cell differentiation, responding to cues provided by the scaffold [72].

Mechanical forces are essential for skeletal homeostasis as they define the bone architecture and drive the differentiation of progenitor cells [150]. Furthermore, the osteogenic potential of MSCs is mechanosensitive, *i.e.* stem cells are affected by the mechanical properties of the cellular microenvironment, particularly its mechanical modulus. MSCs respond to biochemical and physical signals from their extracellular microenvironment niche, which controls stem cell fate [49,151]. ECM stiffness is also known to impact the behavior of many cell lineages. Likewise, the stiffness that a cell can sense is dependent on the elastic modulus and the geometry of the biomaterial [152]. A previous study showed that MSCs do not passively receive signals delivered to them, but actively modify their local environment through the secretion of a proteinaceous ECM and degradation of the surrounding hydrogel matrix to suit their needs. These cell-mediated local modifications affect MSC fate [153]. Another study demonstrated that the matrix stiffness of 3D scaffolds regulated MSCs differentiation potential, showing that high stiffness favored osteogenesis. The MSCs responded differently to hydrogel based-scaffolds with different mechanical moduli by adapting their morphology and distribution of F-actin [49].

As presented in Table 2, hydrogels are rarely used alone, but in combination, to complement the properties of each biomaterial. Different compositions and shapes of scaffolds, cutting-edge technologies, biofunctionalization, and application of signaling molecules to promote cell differentiation, high-quality of biomaterials, and advanced methodologies are reaching favorable outcomes towards osteoblastic differentiation in *in vitro* and *in vivo* researches on hydrogel-based scaffolds for bone regeneration. Nevertheless, despite all of the research and high impact scientific publications, there are still several challenges that prevent hydrogel-based biomaterials for vertical bone augmentation to be feasible for use in clinical applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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2.3ARTICLE 3 - ENHANCED BONE TISSUE REGENERATION WITH HYDROGEL-BASED SCAFFOLDS BY EMBEDDING PARATHYROID HORMONE IN MESOPOROUS BIOACTIVE GLASS

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Enhanced bone tissue regeneration with hydrogel-based scaffolds by embedding parathyroid hormone in mesoporous bioactive glass

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Abstract

Objectives: To evaluate hydrogel-based scaffolds embedded with parathyroid hormone (PTH)-loaded mesoporous bioactive glass (MBG) on the enhancement of bone tissue regeneration in vitro.

Materials and methods: MBG was produced via sol-gel technique followed by PTH solution imbibition. PTH-loaded MBG was blended into the hydrogels and submitted to a lyophilization process associated with a chemical crosslinking reaction to the production of the scaffolds. Characterization, bioactivity, biocompatibility, and osteogenic differentiation analyses of the scaffolds were then performed.

Results: SEM images demonstrated MBG particles dispersed into the hydrogel-based scaffold structure, which was homogeneously porous and well interconnected. EDX and FTIR revealed large amounts of carbon, oxygen, sodium, and silica in the scaffold composition. SEM images obtained after the bioactivity experiment revealed changes on sample surfaces, over the analysed period, indicating the formation of carbonated hydroxyapatite; however, the chemical composition given by EDX remained stable. PTH-loaded hydrogel-based scaffolds were biocompatible for mesenchymal stem cells (MSCs). A high quantity of calcium deposits on the extracellular matrix of MSCs was found for PTH-loaded hydrogel-based scaffolds. μCT images were in accordance with SEM, showing MBG particles dispersed into the scaffolds structure, and a porous, lamellar, and interconnected hydrogel architecture.

Conclusions: PTH-loaded hydrogel-based scaffolds demonstrated consistent morphology and physicochemical properties for bone tissue regeneration, as well as bioactivity, biocompatibility, and osteoinductivity in vitro. The herein presented scaffolds are recommended for future studies on 3D printing.

Clinical relevance: Bone tissue regeneration is still a challenge for several approaches to oral and maxillofacial surgeries; though, tissue engineering applying MSCs, scaffolds, and osteoinductive mediators might help to overcome this clinical issue.

Keywords: Bone regeneration, hydrogel, bioactive glass, parathyroid hormone, mesenchymal stem cells.

1 Introduction

The regeneration of lost tissues, whether by trauma or degenerative diseases, is always complex [1, 2]. The loss of bone dimensions is very common in individuals who have suffered from tooth loss. In general, when these patients seek to restore health, function, and aesthetics through rehabilitation treatment, they have difficulty installing dental implants due to bone loss. Then, it is necessary to clinically apply bone grafts. However, even nowadays, the predictability of bone augmentation is reduced due to the difficulty in regenerating a dynamic and vascularized tissue such as bone. Therefore, the application of scaffolds is recommended [1, 3, 4].

Mesenchymal stem cells (MSCs) act on osteogenesis since they can differentiate into bone-forming cells under proper stimuli [1, 2]. Nevertheless, the activity of MSCs is regulated by interactions with the extracellular matrix (ECM); thus, applying biomaterials with similar performance to ECM is recommended [1]. Hydrogels are polymeric networks consisting of cross-linked hydrophilic components that can imbibe large amounts of biological fluids while remaining insoluble for a period. The high affinity for fluids gives hydrogels physical properties resembling living tissues, such as a soft consistency and low interfacial tension with aqueous media [5, 6]. Hydrogels have emerged as promising biomaterials for bone tissue regeneration applications since they are generally biocompatible, biodegradable, and can mimic ECM architecture; however, they lack mechanical resistance. Therefore, other components should be embedded into their structure to improve scaffolds' mechanical strength [7–9]. In biomineralized tissues, such as bone, the collagen matrix is stiffened by the apatite crystals which act as filler particles [10]. Bioactive glasses have been shown to be excellent biomaterials due to their characteristics of high bioactivity, osteogenic stimulation, angiogenic effect, high biocompatibility, and antibacterial activity induced by ion release [11, 12]. Highly bioactive synthetic glasses bind to bone through the formation of a carbonated hydroxyapatite layer, which mimics the bone mineral phase, resulting in a biological combination between bioactive glasses and bone tissue [11, 12]. The incorporation of bioactive glass into the hydrogel structure not only makes the mechanical properties of the scaffold more similar to that of bone but also participates in the bioactivity of the material.

Scaffolds based on bioceramics and polymers have evolved shifting from a passive role where they are merely accepted by the body to an active role where they respond to environmental conditions or to different types of cues generating suitable integration inside the host tissue

[13]. A variety of signalling molecules have been investigated for their ability to induce and accelerate bone regeneration. However, most of them frequently fail in translational trials due to side effects resulting from the supraphysiological concentrations needed to achieve the desired repair. Parathyroid hormone (PTH) is well known to stimulate bone remodelling and can lead to either bone loss or bone gain depending on the balance between bone resorption and formation [14]. Effective systems to deliver PTH to the desired local site, preserve PTH bioactivity, and induce optimal bone activity might be an encouraging strategy for bone regeneration purposes [14–18]. Herein, we aimed to develop a scaffold gathering the moldability and hydrophilicity of hydrogels, the biocompatibility and bioactivity of bioactive glasses, and the potential of PTH to enhance the osteoinduction of MSCs for bone tissue regeneration in vitro.

2 Material and methods

2.1 Scaffolds preparation

Mesoporous bioactive glass 58S (MBG58S; 58 wt.% SiO_2, 33 wt.% CaO, 9 wt.% P_2O_5) powder was produced via sol-gel technique as previously described [11]. Hydrogel-based scaffolds were prepared by lyophilization associated with a chemical crosslinking reaction. The fabrication process of the hydrogel-based scaffolds and loading of PTH into the mesoporous BG are presented in Fig. 1. Firstly, 5 µg/mL PTH (≥95% HPLC, P7036, Sigma-Aldrich, USA) in 1% acetic acid was loaded to the MBG58S powder. The solution was dried for 24 h. Hydroxyethylcellulose (434965, 90,000 g/mol, Sigma-Aldrich, USA) and sodium alginate (W201502, Sigma-Aldrich, USA) solution (10% w/v) in a 1:1 proportion was prepared by dissolving hydrogel powders in deionized water and the solution was maintained under stirring for 60 min at 60 °C. The PTH-loaded MBG58S powder was then blended with the hydrogel mixture in a proportion of 1.5:1. Separately, MBG58S with no PTH incorporation was blended with the hydrogels and set as a control group. The mixtures were placed in cylindrical molds and kept at −20 °C overnight. The frozen samples were lyophilized at −54 °C for 24 h. The scaffolds were immersed in 5% $CaCl_2$ (C7902, Sigma-Aldrich, USA) for 3 h for chemical crosslinking and washed three times with deionized water. The cross-linked samples were then sterilized through gamma radiation.

2.2 Characterization analyses

The microstructural properties of the scaffolds were determined by using a Scanning Electron Microscope (SEM, JEOL JSM-6390LV, Hitachi, Japan), connected with an X-ray detector (EDX) unit, with 20–25 kV accelerated voltage and image amplification up to 400,000×. Samples were freeze-dried prior to analysis, mounted on aluminium stubs, and sputter-coated with gold-palladium [19–21]. Fourier Transform Infrared (FTIR) Spectroscopy (Cary 660, Agilent, USA) estimations were registered at room temperature in the wavenumber range of 650-4000 cm^{-1}, 20 scans, and resolution of 4 cm^{-1}. This analysis provided the chemical composition of the samples and confirmed the incorporation of the bioactive compound [9, 19, 22].

The mechanical properties of the scaffolds were evaluated by measuring the compression strength. Samples (n = 5) were placed between parallel plates utilizing a component EMIC DL3000 (Instron, Brazil) and compressed with a crosshead speed of 0.1 mm/s and a 50 N load cell at room temperature [9, 19, 21]. Rheological measurements of hydrogels were performed with a MARS III rheometer (HAAKE MARS, Thermo Fisher Scientific, USA) using a parallel plate (25 mm plate diameter, 5 mm gap) in oscillatory mode at 37 °C. Changes of the storage modulus (G') and loss modulus (G") were measured over an oscillation torque range (angular frequency) of 0.1 to 100 rad/s [9, 23]. Swelling of hydrogel-based scaffolds was performed by immersing samples (n = 5) in 24-well plates with 2 mL of phosphate-buffered saline (PBS) at 37 °C for 7, 14, 21, and 28 days. Samples were weighed before immersion and recorded as W_i. After each experimental time, samples were collected, gently placed on filter paper to eliminate the adsorbed water, dried in a vacuum incubator, weighed again (W_f), and returned to the wells of the plate. The PBS was renewed at each experimental time. The swelling ratio was calculated by $(W_i–W_f)/W_f$. Similarly, the degradation (%) of the scaffolds was determined by $(W_i–W_f)/W_i \times 100\%$ and presented as mean ± standard deviation [9, 19, 21].

The release of PTH from the hydrogel-based scaffolds was monitored as a function of incubation time in PBS. Samples (n = 5) were immersed in 24-well plates with 2 mL PBS at 37 °C for 1, 7, 14, 21, and 28 days. At each time interval, 100 µL of the release medium was collected from the wells and measured by spectrophotometry at 450 nm. PBS was renewed at each time interval. A standard curve was determined with known concentrations of PTH. To analyse the PTH release profile, the PTH concentration was re-dimensioned by dividing the absolute amount of PTH released at time *t* (M_t) by the amount of PTH at infinity (M_∞), which

was the equilibrated concentration obtained at the end of the 28-day experimental period, in order to measure the material solubility.

2.3 Bioactivity

To analyse the bioactivity, the samples were immersed in 2 mL of simulated body fluid (SBF) in 24 well-plates and incubated at 37 °C, 90 rpm, for 1, 3, and 7 days. SBF solution was prepared following Kokubo's method [24]. After each experimental time, samples were removed from the SBF, washed twice with deionized water, refrigerated for 12 h at −20 °C, and then lyophilized for 12 h. Dried samples were analysed using SEM (**JEOL JSM-6390LV**) to study the morphology of deposited apatite and EDX to determine the chemical composition [25]. The negative control group was pure scaffolds immersed in deionized water following the same described procedures for SBF immersion. The experiments were run in triplicate.

2.4 Biocompatibility

Stem cells from human exfoliated deciduous teeth (SHED) were cultured in Minimum Essential Medium α (α-MEM, Lonza, Switzerland) with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA). 48-well plates were used, 5×10^4 cells per well were incubated at 37 °C and 5% CO_2 for 4 h, and then the samples (n = 3) were placed into the wells. Cytotoxicity was analysed by Thiazolyl Blue Tetrazolium Bromide (MTT; M2128, Sigma-Aldrich, USA) colourimetric test [21]. Experimental times were on days 1, 3, and 7. At each time interval, samples were treated with MTT solution in a proportion of 1:5 with the culture media and incubated for 4 h at 37 °C. DMSO was used to solubilize the formazan crystals and the absorbances were read at 540/630 nm (CLARIOstar Plus, BMG Labtech, Germany). Tests were performed in triplicate.

2.5 Osteogenic differentiation

SHED were cultured in α-MEM with 10% FBS (standard medium). 48-well plates were used, 5×10^3 cells per well were incubated at 37 °C and 5% CO_2 for 24 h, and then the samples (scaffold and PTH-loaded scaffold groups; n = 5 each) were placed into the wells. Additionally, the following control groups were evaluated: cell control- 5×10^3 cells per well cultured in standard medium, and osteogenic control- 1×10^4 cells per well cultured in osteodifferentiation medium (StemPro Osteogenesis Differentiation Kit, Gibco, Thermo Fisher Scientific, USA). The following assays were performed:

2.5.1 Von Kossa staining

After 28 days of cell culture, nodules of mineralization in the extracellular matrix of SHED were stained using von Kossa staining. Samples were removed and cells were washed with PBS, fixed with 4% paraformaldehyde for 60 min at room temperature, and washed with ultrapure water. Cells were then treated with 1% silver nitrate solution and incubated for 30 min, protected from light.

2.5.2 Alizarin Red S staining

Calcium deposits in the extracellular matrix of SHED were analysed by Alizarin Red S assay after 28 days of cell culture. Cells were treated with 0.3% alizarin red solution and incubated for 30 min. Cells were then washed with ultrapure water and the results were observed and photographed from the conventional light microscope.

2.5.3 Micro-computed tomography (μCT) assay

After 28 days of cell culture, micro-computed tomography (μCT) images were obtained to analyse the sample's internal mineralization process. SHED were seeded over the already placed samples (n = 3) soaked in standard medium. 5×10^4 cells per sample, using a 48-well plate, were incubated at 37 °C and 5% CO_2. After 28 days, samples were washed with PBS and fixed in 4% paraformaldehyde for 60 min at room temperature. Samples were dried with sequential growing concentrations of ethanol and scanned using a SkyScan1272 (Bruker, USA) μCT scanner. MicroView software (GE Healthcare, USA) was used for visualization and analysis. Two-dimensional images were obtained from μCT cross-sectional images to evaluate the mineral formation and ImageJ (NIH, USA) software was used to calculate the mean grey value and samples area.

2.7 Statistical analyses

Mechanical compression, swelling, and degradation data were analysed by two-way ANOVA followed by Sidak's multiple comparison test. Mean grey values and the area of μCT were analysed by paired t-test. Biocompatibility and gene expression of samples were analysed by one-way ANOVA followed by Tukey's multiple comparison test. All data were run using Prism 8 software (GraphPad, USA). Differences were considered statistically significant when $p < 0.05$.

3 Results and discussion

The development of bioactive scaffolds to increase bone tissue regeneration has become a strategic field of tissue engineering research [21]. Applying temporary and porous three-dimensional scaffolds for the delivery and integration of cells and/or active substances at the repair site is a critical approach for tissue regeneration. The growth and differentiation of tissues in such bone substitutes are also directly affected by the microstructure and bioactivity of the selected materials. The remarkable characteristics of hydrogels, including tuneable physicomechanical properties, resemblance to the native extracellular matrix, and hydrophilicity, allow cell proliferation and differentiation and offer opportunities to overcome the tissue vascularization [21]. Herein, we produced and tested in vitro the properties of a hydrogel-based scaffold. Hydroxyethylcellulose and sodium alginate were blended and crosslinked to produce a hydrophilic bone-like structure, while the bioactive glass was added to provide bioactivity and mineral composition to the scaffold. Hydroxyethylcellulose is a biocompatible, non-ionic carbohydrate polymer with surface-active properties that can function as a "protective" colloid, compatible with an extensive variety of water-dissolvable polymers [19, 26]. In turn, sodium alginate is biocompatible, bioadhesive, biodegradable, has low toxicity, and has non-immunogenic effects. Also, sodium alginate has negatively charged groups (carboxylates) where biogenic hydroxyapatite can be deposited (nucleation sites) [13]. On the other hand, bioactive glass is a promising bioceramic for bone regeneration due to its high biocompatibility and bioactivity, angiogenic effect, and antibacterial activity induced by ion release, particularly Ca^{2+}, Mg^{2+}, and PO_4^{3-} [27–31]. These materials were proposed to overcome the disadvantages of ceramic materials used alone in the filling of bone defects [13]. The incorporation of active substances in tissue-engineered scaffolds can enhance osteoinductivity and promote bone repair [32]. Thus, PTH was embedded into the mesoporous bioactive glass to add osteoinductivity to the composite scaffold. PTH is an 84 amino acid and a key regulator of calcium homeostasis in the body [16]. Since a chronically high PTH dosage does not necessarily have catabolic effects on bone and a mild PTH dosage may be beneficial for trabecular bone, PTH local delivery may improve bone regeneration [16, 17]. Also, the PTH delivery system improvements may optimize the bone healing response, while decreasing or eliminating systemic PTH exposure. Thus, characterization of the PTH release and the degradation of the materials used to deliver PTH were performed in the present study.

3.1 Characterization analyses

SEM, EDX, and FTIR results of hydrogel-based scaffolds are presented in Fig. 2. SEM images demonstrate bioactive glass particles dispersed into the hydrogel-based scaffolds structure, whereas hydrogel-based scaffolds embedding PTH showed certain agglutination of the bioactive glass particles. The architecture of the hydrogel, however, was homogeneously porous and interconnected in both analysed groups. EDX findings revealed large amounts of carbon (C), followed by oxygen (O), sodium (Na), and silica (Si), as expected due to the composition of the bioactive glass associated with the hydroxyethylcellulose and sodium alginate hydrogels. Interestingly, the %wt. of Si was higher for PTH-loaded scaffolds. The bands revealed by FTIR confirm the chemical composition predetermined by EDX. Additionally, the FTIR bands found herein are comparable to those found for previous studies of a bilayered hydrogel of gellan gum and demineralized bone particles for osteochondral regeneration [33], as well as a novel alginate/hydroxyethyl cellulose/hydroxyapatite composite scaffold for bone regeneration [19].

Mechanical compression and elasticity modulus of hydrogel-based scaffolds are presented in Fig. 3. Compression stress was higher for PTH-loaded scaffolds up to about 15% applied strain ($p < 0.001$ at 0% and 10% applied strain). Conversely, from 20% up to 50% strain, scaffolds without PTH showed higher compression stress ($p < 0.05$ at 20% applied strain and $p < 0.001$ at 30%, 40%, and 50% applied strain). The compressive modulus of 0.06 MPa reached for both scaffolds under 50% strain was in accordance with a recent study of dextran composite hydrogel-based scaffolds reinforced with 20 wt% β-TCP [21] but higher than a study of injectable polysaccharide hydrogel embedding hydroxyapatite and calcium carbonate [9]. This is still considered a low compressive modulus compared to the desired value for natural bone [34]; however, it does not mean that the new bone will have a different compression module from native bone, but that post-operative care should be maintained until the end of the bone regeneration.

Rheological measurements revealed similar storage (G') and loss (G'') moduli for both scaffolds (with and without PTH loading). The G′ was greater than G″ by approximately an order of magnitude for both comparative samples over the entire range of frequency, which indicates the stability of the three-dimensional (3D) network in the hydrogel system via the Schiff-base reaction. Compared to a previous study of injectable polysaccharide hydrogel incorporating different percentages of hydroxyapatite and calcium carbonate [9], our study

presented higher elasticity moduli in a range over 10^3 Pa, which is suitable for non-injectable 3D-printed personalized scaffolds.

The swelling behaviour, degradation, and PTH release of hydrogel-based scaffolds are presented in Fig. 4. The swelling ratio was similar for both groups on day 7 but significantly higher for the PTH-loaded scaffold group on days 14, 21, and 28 of the experiment ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively) (Fig. 4A). Accordingly, weight percentage degradation was similar for scaffolds and PTH-loaded scaffolds on days 7, 14, 21, and 28 ($p = 0.8247$, $p = 0.3864$, $p = 0.6970$, and $p = 0,7239$, respectively), but the weight loss significantly increased over the course from day 7 up to day 28 of degradation experiment (Fig. 4B).

The degradation of the hydrogel-bioceramic composites in PBS is high under incubation [35]. Similar to our findings, Choi et al. (2020) [33] exhibited a degradation increased level until 21 days into the experiment and then reached equilibrium. The rapid rate of decomposition in the initial state, i.e., up to day 14, was attributed to the escape of the residual matrix and the hydrophilicity of hydrogels.

Swelling is the ability by which scaffolds uptakes and preserves water within their structure, the water uptake capacity is one of the most important properties of hydrogels. Therefore, it is an important feature for developing suitable tissue engineering constructs for the regeneration of bone defects [19]. Hydrogel swelling behaviour plays a fundamental role in cell migration, proliferation, and angiogenesis. However, the balance between swelling behaviour and physicomechanical properties of hydrogels should be achieved to offer a successful bone tissue regeneration [19, 21]. Generally, the driving force for the water uptake of a hydrogel includes at least three components, i.e., internal elastic force of polymer chains in the network, polymer-water mixing, and osmotic pressure. The interplay of three balancing forces determines the equilibrium swelling ratio. Mixing pressure is due to the solvation of the network, while elastic pressure is stretching polymer chains under the influence of solvation. The ionic pressure originates from the mobility of ions between the hydrogel matrix and the medium. The internal elastic force of polymer chains in the hydrogel network is strongly dependent on the crosslinking density, and interactions between polymeric chain segments and solvent molecules. Thus, loosely crosslinked hydrogels fail to retain water while highly cross-linked polymer networks do not swell [21].

The swelling kinetics was analysed with the Fickian kinetics model to get an insight into the water uptake behaviour of scaffolds. Fick's model was applied based on Equation 1:

$$Wt/Wf = kt^n,$$

where t is the swelling predetermined time, k is the initial swelling rate constant, and n is the diffusion index calculated from the slopes of ln(Wt/Wf) as a function of ln(t) (Fig. 4C). At $n \leq 0.5$, Fickian diffusion plays the decisive role (rate of diffusion << polymer chains relaxation), while $0.5 < n < 1$ corresponds to non-Fickian diffusion in which the rate of diffusion and the rate of polymer chains relaxation are comparable. In $n = 1$, the relaxation of polymer chains plays the leading role (rate of diffusion >> polymer chains relaxation) [21]. In the present study, non-Fickian diffusion was found for both groups (scaffolds and scaffolds+PTH). However, the swelling kinetics based on Fick's model showed a relatively low correlation coefficient (R^2), thus the second-order correlation Schott's model was applied based on Equation 2:

$$(t/Wt) = (1/k_{is}) + (1/W\infty)t,$$

where Wt is the swelling at time t, W∞ is the theoretical equilibrium swelling, and k_{is} is the initial swelling rate constant (Fig. 4D). Higher correlation coefficients were obtained for this model (R^2 >0.99). The initial swelling rate in Schott's model is related to the rate of polymer chains relaxation, which is affected by the following factors: the hydrophobicity/hydrophilicity, the degree of crosslinking, the rigidity/flexibility, the amorphous regions/crystalline domains, and the thickness of the hydrogel [21]. The initial swelling rate constant (k_{is}) was higher for pure scaffolds than for scaffolds embedded with PTH. According to Zhao, the high crosslinking density increases the interaction between polymer chains and should reduce the k_{is} values [36]. The drug release profile of PTH-loaded hydrogel-based scaffolds revealed an expressive release of PTH from the scaffolds from day 1 to day 14, then reached an equilibrium until day 28 (Fig. 4E). The release of the drug from hydrogels (or other polymeric associations) involves various chemical, physical, and biological interactions, which hinders the theoretical prediction of the release process [13].

3.2 Bioactivity and biocompatibility

Bioactivity (SBF) and biocompatibility (MTT) results are depicted in Fig. 5. SEM images obtained after the bioactivity experiment reveal changes on sample surfaces over the analysed period, indicating the formation of carbonated hydroxyapatite. However, the chemical composition given by EDX analyses remains stable over control on days 1, 3, and 7. Bioactivity assay is an obligatory step to propose any material as a potential bone substitute [13]. It can be evaluated *in vitro* by exposing the biomaterials to conditions that mimic the implant environment (i.e., ionic concentration, pH, and temperature) via soaking in the SBF at 37 °C

[19, 24]. The development of apatite layers in the SBF solution had been regarded as an indication of bioactivity for bioceramics. The construction of a bone-like apatite layer in a scaffold is the precondition for its osteoinduction [25, 33, 37]. Bioactive glasses are known to bind to the bone by forming a layer of carbonated hydroxyapatite, which mimics the mineral phase of bone [27, 38].

Both hydrogel-based scaffolds and PTH-loaded hydrogel-based scaffolds were biocompatible for SHED with cell viabilities similar to the control group on 1, 3, and 7 days of cell culture. Cytotoxicity of hydrogels is not expected, as already seen in many previous studies [19, 21, 25, 32, 33]. However, our biocompatibility findings also revealed that the PTH dose embedded into the scaffolds did not lead to cytotoxicity.

3.3 Osteogenic differentiation

Von Kossa and alizarin red stainings and μCT images are presented in Fig. 6. Von Kossa methodology images revealed nodules of mineralization for hydrogel-based scaffold and PTH-loaded hydrogel-based scaffold groups in a proportional quantity, reduced nodules of mineralization for the osteogenic control group, and no nodules in the ECM of the cell control group. Choi et al. (2020) [33] also applied von Kossa methodology and detected nodules of mineralization into the hydrogel of gellan gum and demineralized bone particles bilayer scaffold. Similarly, Frassica et al. (2019) [32] applied von Kossa staining as a visual indicator of matrix mineralization and revealed considerable enhancement in calcium deposition of a diacrylate poly(ethylene glycol) hydrogel-based scaffolds after 14-day culture with human bone marrow stem cells.

Alizarin red staining revealed a high quantity of calcium deposits for PTH-loaded hydrogel-based scaffold and osteogenic control groups, reduced calcium deposits for hydrogel-based scaffold group, and no calcium deposits for the cellular control group. μCT images are in accordance with the SEM images, showing bioactive glass particles dispersed into the scaffolds structure and porous, lamellar, and interconnected hydrogel architecture in both analysed groups. Mean grey values were similar for both hydrogel-based scaffold and PTH-loaded hydrogel-based scaffold groups, while the calculated mean area was higher for PTH-loaded hydrogel-based scaffold than hydrogel-based scaffold group.

4 Conclusions

In summary, the morphology of the scaffolds presented an interconnected porous structure as desired for bone regeneration. The chemical composition was compatible with the applied raw materials and favourable for extracellular matrix mineralization especially due to the presence of Ca and Si. Also, the mechanical and rheological properties of scaffolds showed sufficient mechanical character to provide a temporary structure for reconstructing new bone tissue. The weight loss and swelling of the hydrogels showed stability over time. Additionally, the bioactivity in the SBF solution led to the formation of carbonated hydroxyapatite due to the bioactive glass embedded into the hydrogel structure. The PTH release from scaffolds structure was gradual and it did not influence the viability of SHED. Additionally, PTH-loaded hydrogel-based scaffolds led to the mineralization of the SHED extracellular matrix after 28 days of cell culture.

The proposed PTH-loaded hydrogel-based scaffolds demonstrated morphology and physicochemical properties, bioactivity, biocompatibility, and osteoinduction capacity to be used in bone tissue engineering. Therefore, the herein presented scaffolds are recommended for future studies on 3D printing. Furthermore, as we have drawn the conclusions of this study based on the data obtained in vitro, future animal models will still be required to validate all our results in vivo.

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Compliance with Ethical Standards

- Conflict of Interests: The authors declare no conflicts of interest.

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- Ethical Approval: Not applicable.

- Informed Consent: Not applicable.

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Figure captions

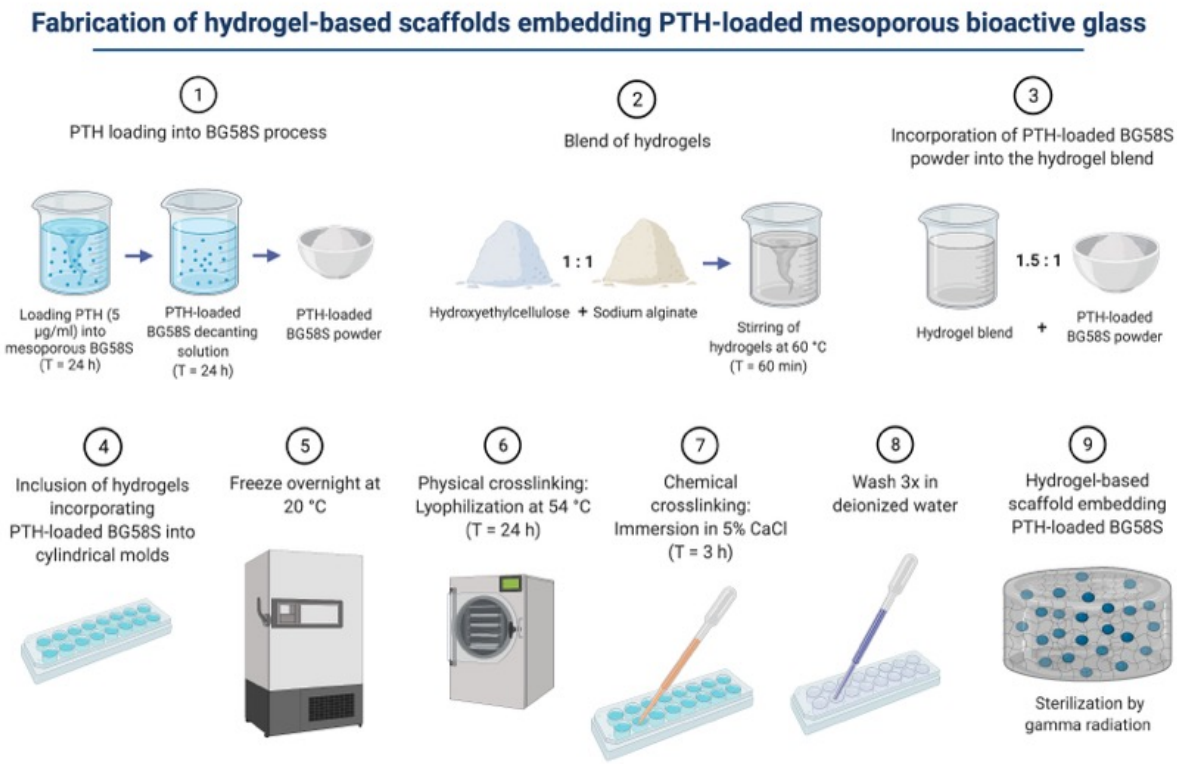


Fig. 1 Fabrication process of hydrogel-based scaffolds embedding PTH-loaded mesoporous bioactive grass. PTH: Parathyroid hormone. BG58S: Bioactive glass 58S composition. T: time. Created with BioRender.com

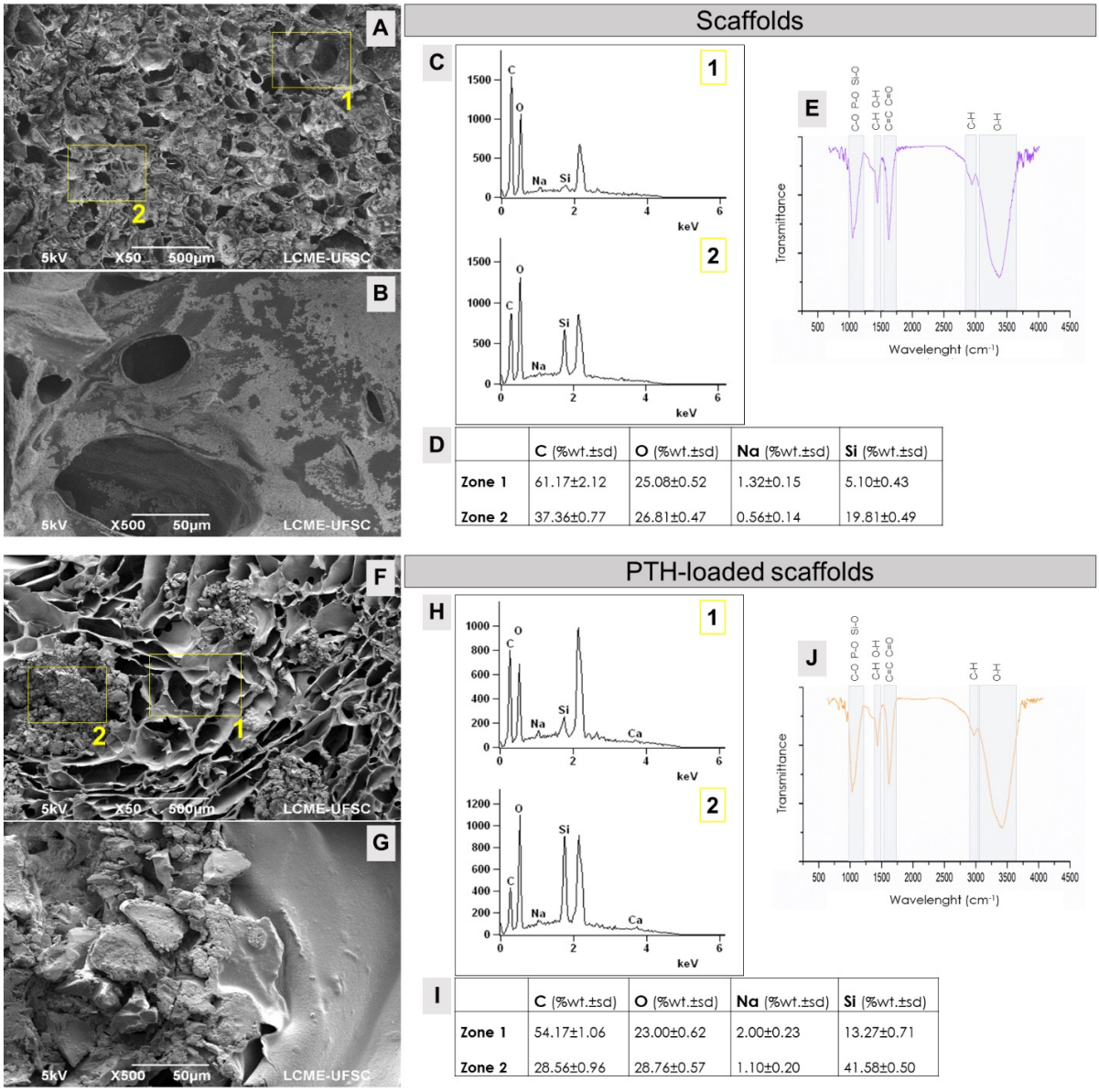


Fig. 2 Hydrogel-based scaffold physical and chemical characterization. A-B) Scanning Electron Microscope (SEM) images of the hydrogel-based scaffold at (A) X50 and (B) X500 magnifications (500 and 50 µm scale bars, respectively); C) Energy Dispersive X-Ray Spectrometry (EDX) graph of hydrogel-based scaffold and (D) weight percentage of chemical composition; E) Fourier Transform Infrared Spectroscopy (FTIR) of hydrogel-based scaffold presenting the main chemical bands under spectra; SEM images of hydrogel-based scaffold embedding PTH at (F) X50 and (G) X500 magnifications (500 and 50 µm scale bars, respectively); EDX graph of hydrogel-based scaffold embedding PTH (H) and (I) weight percentage of chemical composition; J) FTIR of hydrogel-based scaffold embedding PTH presenting the main chemical bands under spectra. C: Carbon; O: Oxygen; Na: Sodium; Si: Silicon

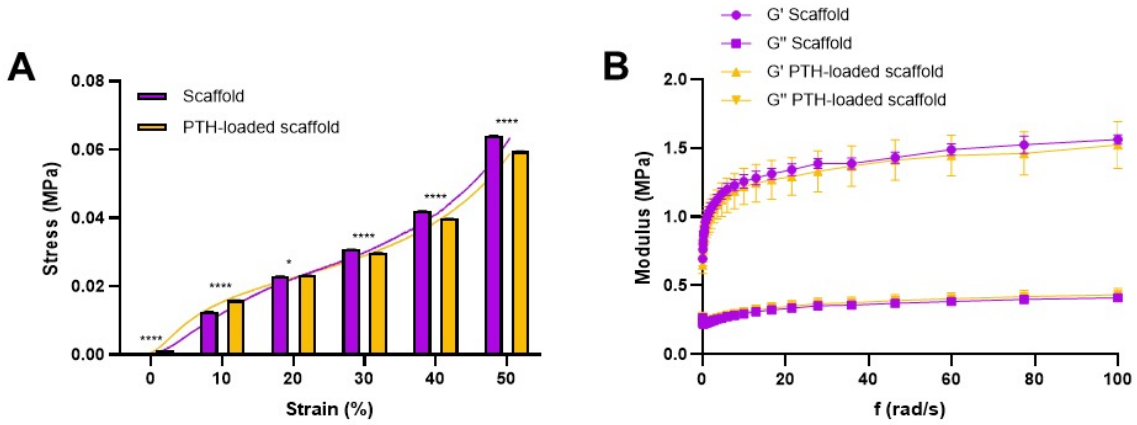
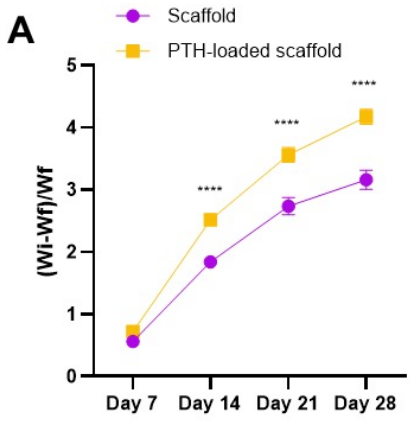
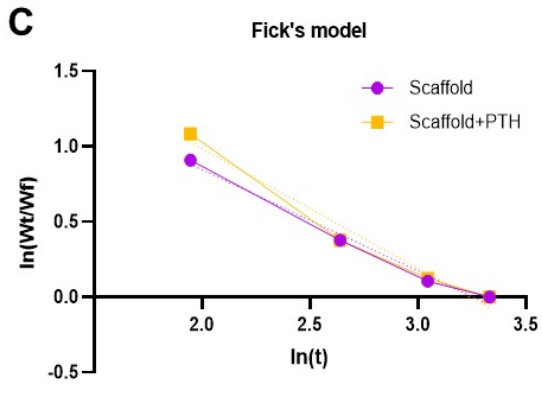


Fig. 3 Hydrogel-based scaffolds mechanical characterization. A) Compression stress as a function of the applied strain. Values reported are an average of n = 6, ± standard deviation. Statistical differences were found between the scaffold and PTH-loaded scaffold groups ($p < 0.05$(*) and $p < 0.0001$(****)). B) Storage modulus (G') and loss modulus (G'') values as a function of the angular frequency (f) of rheological analyses. Values reported are an average of n = 3, ± standard deviation

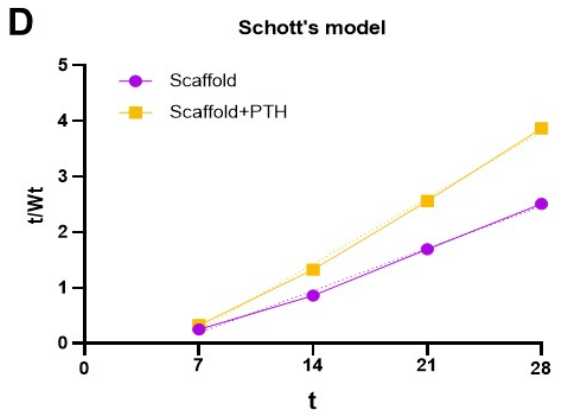


B

	Scaffold	PTH-loaded scaffold
Day 7	34.92±9.64[a]	39.92±10.88[a]
Day 14	61.75±11.62[b]	70.35±6.02[b]
Day 21	70.86±8.25[bc]	76.98±5.16[b]
Day 28	73.78±7.71[c]	79.67±5.21[b]



	k (mg/mg·t)	n	R^2
Scaffold	8.9298	0.6719	0.9876
Scaffold+PTH	13.1169	0.7946	0.9724



	k_{is} (mg/mg·t)	$W\infty$ (mg/mg) (%)	Wf (mg/mg) (%)	R^2
Scaffold	1.74886	1.315443	0.1116	0.9952
Scaffold+PTH	1.05966	0.843455	0.0724	0.9964

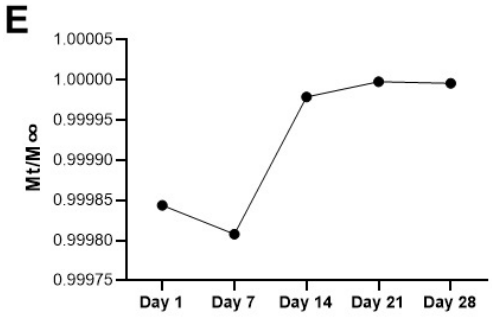
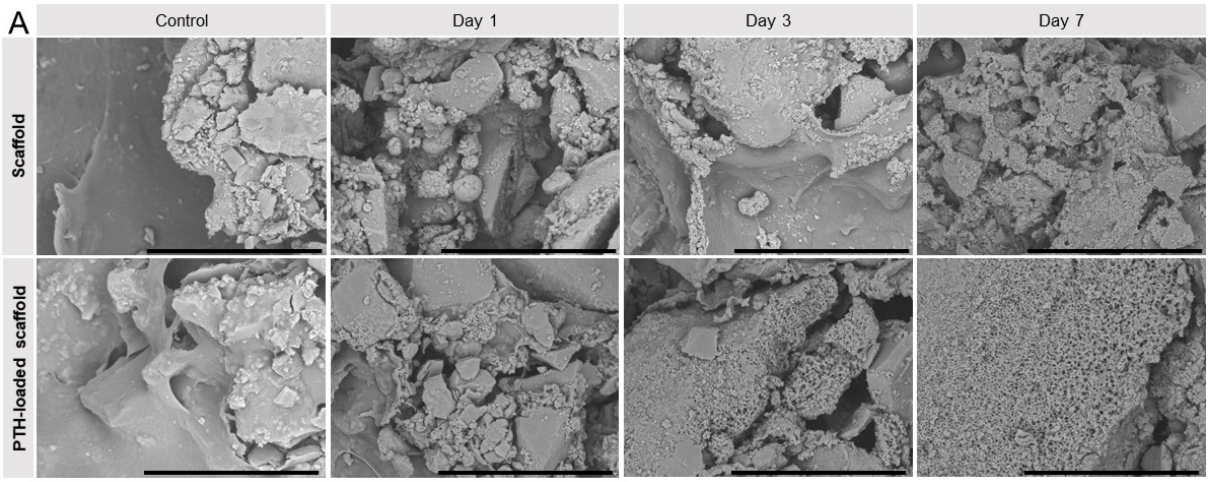


Fig. 4 Hydrogel-based scaffolds swelling behaviour. A) Swelling ratio of hydrogel-based scaffolds on days 7, 14, 21, and 28. Values reported are an average of n = 6, ± standard deviation. Statistical differences were found between the scaffold and PTH-loaded scaffold groups ($p < 0.0001$(****)). B) Weight percentage degradation of hydrogel-based scaffolds on days 7, 14, 21, and 28. Values reported are an average of n = 5, ± standard deviation. No statistical difference was found between the scaffold and PTH-loaded scaffold groups at the same time point, while the statistical difference was observed

over the course of the experimental times (different superscript letters represent statistical differences). C) Fick's first order (Eq. 1) swelling kinetics model of the swelling data was fit. The embedded table presents the swelling parameters obtained from Fick's model (the regression equation is y = A + Bx, where y = ln (Wt/Wf), A = ln (*k*), B = *n*, x = ln (t)). D) Schott's second order (Eq. 2) swelling kinetics model of the swelling data was also displayed. The embedded table presents the swelling parameters obtained from Schott's model (the regression equation is y = A + Bx, where y = t/Wt, A = $1/k_{is}$, B = $W\infty$, x = t). E) PTH release profile of PTH-loaded hydrogel-based scaffolds at days 1, 7, 14, 21, and 28. Values reported are calculated by dividing the absolute amount of PTH released at time *t* (M*t*) by the amount of PTH at infinity (M∞), which was the equilibrated concentration obtained at the end of the 28-day experimental period



B

Scaffold				
	Control	Day 1	Day 3	Day 7
Elements	% wt. ±sd			
C	21.880 ± 0.990	18.652 ± 1.974	20.585 ± 1.564	15.662 ± 2.201
Si	13.009 ± 0.339	9.780 ± 0.402	8.563 ± 0.333	14.383 ± 0.605
Ca	13.255 ± 0.371	12.904 ± 0.520	14.993 ± 0.718	13.013 ± 0.607
P	0.809 ± 0.279	2.038 ± 0.340	2.629 ± 0.343	2.463 ± 0.463
PTH-loaded scaffold				
	Control	Day 1	Day 3	Day 7
Elements	% wt. ±sd			
C	26.508 ± 1.197	24.321 ± 1.873	18.871 ± 1.854	13.480 ± 2.048
Si	14.248 ± 0.416	11.205 ± 0.444	14.947 ± 0.530	22.833 ± 0.733
Ca	9.192 ± 0.354	10.400 ± 0.457	10.765 ± 0.458	9.696 ± 0.420
P	-	-	1.124 ± 0.347	0.817 ± 0.394

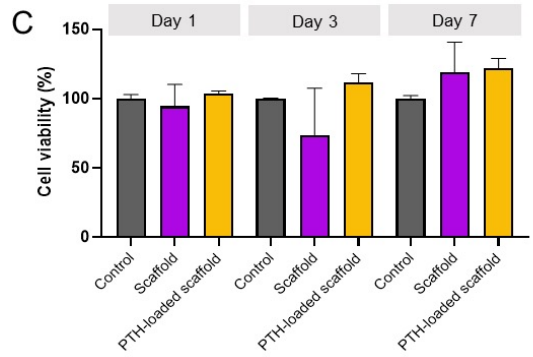


Fig. 5 A) Scanning Electron Microscope (SEM) images analysing the bioactivity of scaffolds using SBF on days 1, 3, and 7. Control was the scaffold immersed in PBS for 3 days. B) Chemical composition (weight % ± standard deviation) of samples given by EDX analyses on days 1, 3, and 7. Control was the scaffold immersed in PBS for 3 days. C) Biocompatibility of scaffolds on days 1, 3, and 7. Control was the cells seeded at the same density but with no scaffold influence. Values reported are an average of n = 3 ± standard deviation. No significant difference was found among SHED control group, scaffold, and PTH-loaded scaffold regarding cell viabilities

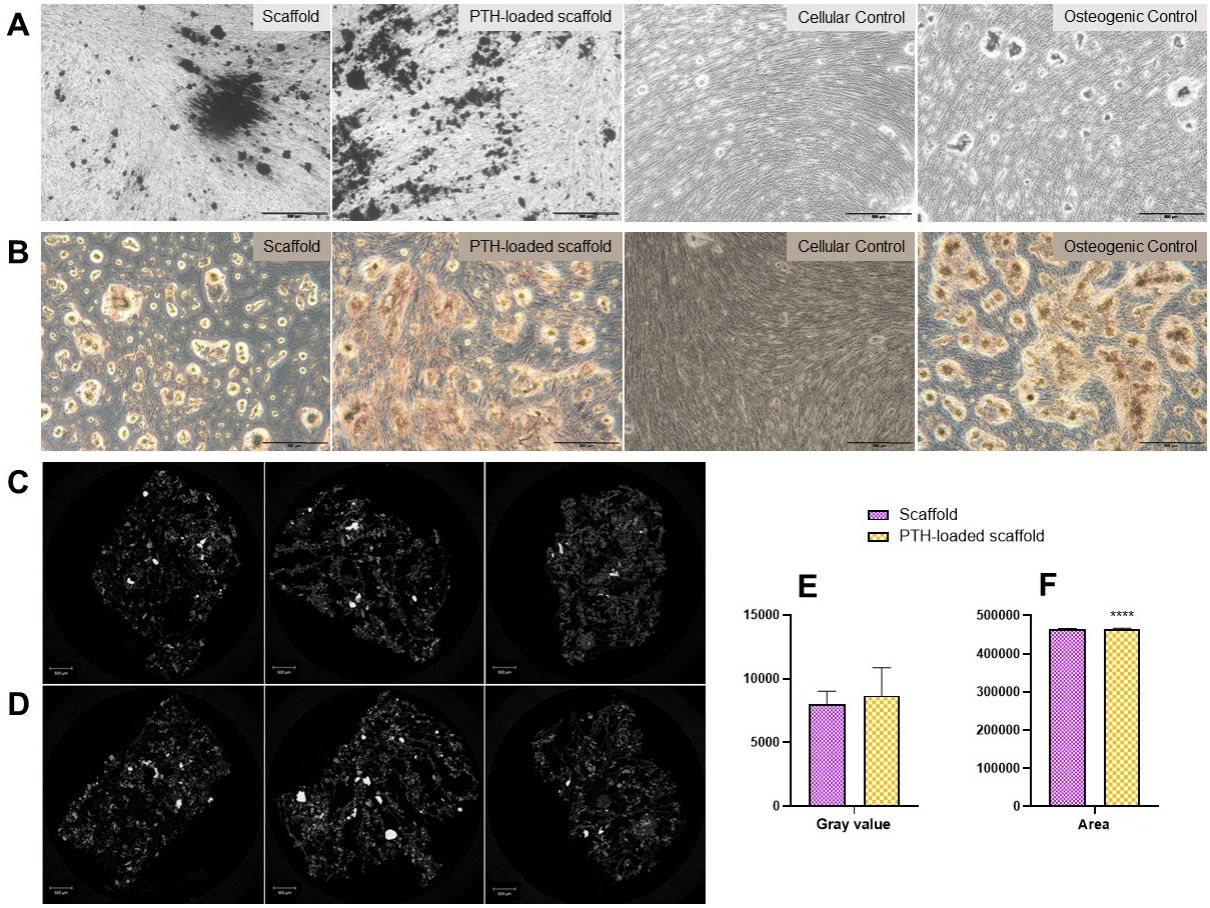


Fig. 6 A-B) Mineralization of the extracellular matrix of stem cells from human exfoliated deciduous teeth (SHED) on day 28. 500 µm scale bars. (A) Black/grey marks represent the nodules of mineralization under von Kossa staining, while (B) brown agglutinations are the alizarin red-stained calcium deposits. Micro-computed tomography (µCT) of (C) scaffolds (n = 3) and (D) PTH-loaded scaffolds (n = 3) on day 28 of SHED culture. 500 µm scale bars. E) Grey values of the samples were calculated based on µCT images. Values reported are an average of n = 3 ± standard deviation. No significant difference was found between the scaffold and PTH-loaded scaffold groups (p = 0.6146). F) Area (cm^2) of the samples calculated based on µCT images. Values reported are an average of n = 3 ± standard deviation. A significant difference was found between the scaffold and PTH-loaded scaffold groups (p <0.0001)

2.4 ARTICLE 4 - PYROPTOSIS-MEDIATED PERIODONTAL DISEASE

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Review

Pyroptosis-Mediated Periodontal Disease

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Abstract: Pyroptosis is a caspase-dependent process relevant to the understanding of beneficial host responses and medical conditions for which inflammation is central to the pathophysiology of the disease. Pyroptosis has been recently suggested as one of the pathways of exacerbated inflammation of periodontal tissues. Hence, this focused review aims to discuss pyroptosis as a pathological mechanism in the cause of periodontitis. The included articles presented similarities regarding methods, type of cells applied, and cell stimulation, as the outcomes also point to the same direction considering the cellular events. The collected data indicate that virulence factors present in the diseased periodontal tissues initiate the inflammasome route of tissue destruction with caspase activation, cleavage of gasdermin D, and secretion of interleukins IL-1β and IL-18. Consequently, removing periopathogens' virulence factors that trigger pyroptosis is a potential strategy to combat periodontal disease and regain tissue homeostasis.

Keywords: pyroptosis; immune response; inflammation; periodontal disease; periodontitis

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1. Introduction

In clinical practice, clinicians frequently face situations where the periodontal or peri-implant tissues overreact to a stimulus promoted by dental materials or even do not respond to therapies, leading to inflammation. In these situations, the claim is that the body is not accepting the treatment, rehabilitation, or therapy [1–4]. Indeed, it is very likely that processes are occurring inside the cells to cause such exacerbated inflammation. However, the root cause of the inflammatory processes and the activated cell pathways that culminate in tissue damage are beginning to be understood. There may be some novel inflammation pathways leading to exacerbated tissue damage that requires the attention of researchers and clinicians.

Pyroptosis is a process of cellular self-destruction mediated by caspases. Thus, when pathological or damaging factors stimulate cells, they promote the formation of inflammasomes. Pyroptosis is chiefly mediated by the activation of caspase-1 by the NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome [5,6]. Caspase-1 cleaves gasdermin D (GSDMD), resulting in cell membrane perforation through the release of the GSDMD N-terminal fragment [5]. This is known as the canonical inflammasome activation of pyroptosis. The non-canonical activation of pyroptosis occurs via the activation of caspases-4 and -5 in humans, or caspase-11 in mice [7–9], which also cleave the GSDMD. Thus, the mechanisms of pyroptosis involve different major signalling pathways, all activating the downstream of GSDMD. Finally, cytoplasmic molecules, such as interleukins-1β (IL-1β) and -18 (IL-18), are released from the pores formed by GSDMD and trigger a robust inflammatory response (Figure 1) [2,9,10]. Thus, the occurrence of pyroptosis can be determined by a combination of markers, including the activation of

caspases-1, -4, -5, and -11, the cleavage of GSDMD, and the maturation and release of IL-1β and IL-18 [9].

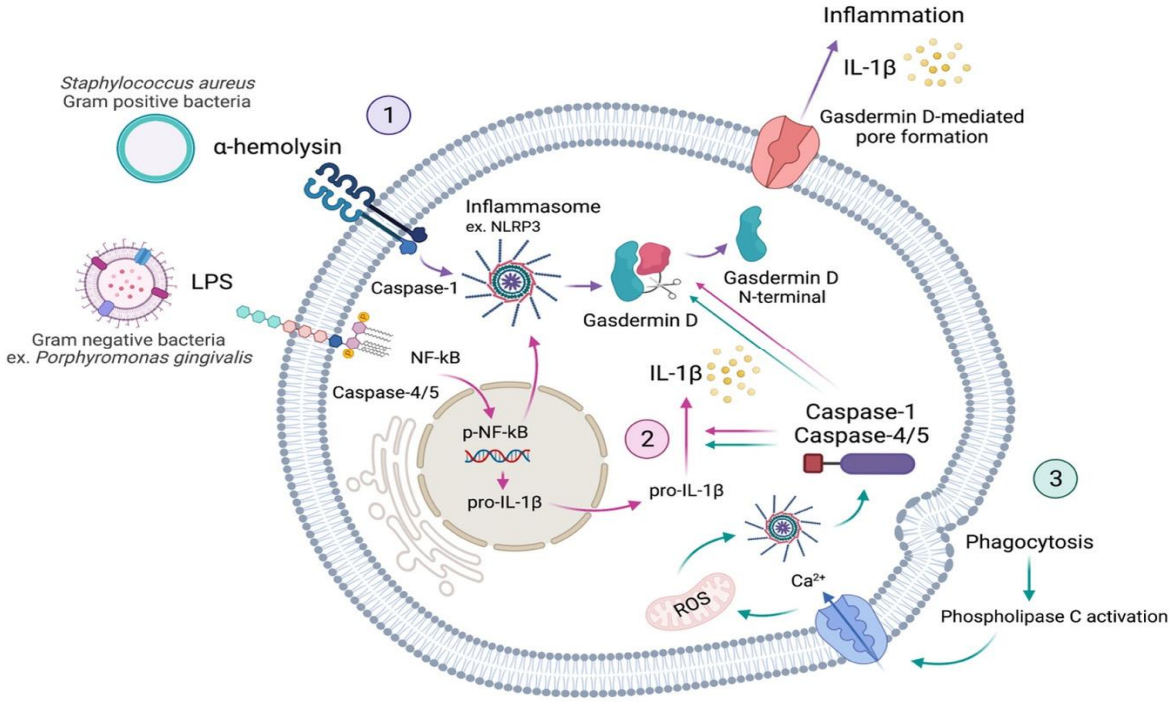


Figure 1. Schematic representation of the pyroptosis activation in a cell. Virulence factors, such as α-hemolysin and LPS, activate inflammasome immune responses by activating caspases. Activated caspase-1 (canonical) and caspases-4/5 (non-canonical) recognise and cleave gasdermin D. The N-terminal fragment, resulting from Gasdermin D cleavage, is responsible for pore formation on the cell membrane. These pores allow the release of interleukins and thus induce the inflammation process called pyroptosis (1, purple arrows). Additionally, activated inflammasomes are responsible for cleaving caspases into subunits, which induce the maturation of pro-inflammatory cytokines, such as pro-interleukin-1β (pro-IL-1β). Activated IL-1β can induce the expression of various genes, including RANKL (receptor activator of p-NF-κB ligand) and activate pyroptosis (2, pink arrows). Furthermore, phagocytosis can lead to pyroptosis through the activation of phospholipase C, which allows the intake of calcium (Ca^{2+}), provoking the production of mitochondrial reactive oxygen species (ROS), which can also activate the inflammasome route (3, green arrows) [5,11].

With the discovery of NLRP3, GSDMD, and caspase-1 as significant drivers of pyroptosis, small molecule inhibitors that block these factors functions are expected to emerge for possible treatment of inflammatory conditions [12]. Therefore, this focused review aims to discuss pyroptosis as a catabolic mechanism present, in the oral environment, in the face of exposure to damaging factors, as well as propose it as a target for periodontal therapies. This review further highlights the importance of oral hygiene to avoid accumulating virulence factors that drive pyroptosis in the periodontium.

2. Methods

A bibliographical search was performed on MEDLINE/PubMed (via National Library of Medicine) using the following search terms: (innate immune system or immune system or inflammation or inflammatory response or inflammatory process) and (cytokine or interleukin) and (cell death or proptosis) and (virulence factor or virulence factors or hemolysin or nigericin or LPS or lipopolysaccharide) and (inflammasomes or caspase or gasdermin) and (periodontal disease or periodontitis or (periodontal or periodontally or

periodontics or periodontic or periodontitis) and (therapeutics or therapies or therapy)). Additionally, a manual search was performed considering the references within the selected articles. The inclusion criteria involved English language articles published up to July 2021, reporting systematic reviews, literature reviews, and in vitro, in vivo, preclinical, and clinical studies on the cell events occurring under the stimuli of different virulence factors that lead to pyroptosis and inflammation. The following exclusion criteria were considered: case reports, protocols, short communications, personal opinions, letters, posters, conference abstracts; full text not available; duplicate data (e.g., dissertations/thesis in which correspondent published articles were available).

Regarding the title and abstract, the evaluation of the potentially relevant articles was accomplished. Selected articles were individually read and analysed, considering the aim of this review. The retrieved variables considered for this review were: authors' names; journal; publication year; study design; methods; statistical analyses. A software (Zotero, George Mason University, Fairfax County, VA, USA) was used to manage references. A two-phase selection process was performed. In the first phase, a title and abstract reading were conducted to identify potentially eligible studies. In the second phase, full-text reading of eligible articles was carried out. The following data regarding included studies were recorded: study characteristics (author, year, journal of publication), type of study, methods, and main findings/outcomes.

3. Results

The initial search strategy retrieved 23 potential studies published from December 2005 to June 2021. The year 2017 was the one that resulted in the most publications (seven studies), followed by 2021 (four studies), and 2016 and 2020 (three studies each year). The manual search resulted in additional information for the present review. One study was a literature review [8], while the 22 remaining articles were in vitro and/or in vivo studies (Tables 1 and 2).

In the first phase, the studies that analysed other pathways for cell death, such as apoptosis, were excluded from the full reading and inclusion in this review (Table 3). In the second phase, no articles were excluded from the full-text reading. Generally, articles followed similar study designs, methods, purposes, and outcomes. Methods included cell or animal stimulation for pyroptosis, followed by analyses of cell viability, cell morphology, histology, expression of pyroptosis-related proteins, interleukins, and genes. The studies that did not mention pyroptosis but analysed caspases-1, -4, -5, or -11, which are related to pyroptosis, were included in the review [6,7].

Table 1. Included experimental articles.

	Authors	Year	Journal	Study Type	Methods	Outcomes	Title and Reference
1	Zhang X, He S, Lu W, Lin L, Xiao H.	2021	In Vitro Cellular & Developmental Biololy-Animal	In Vitro	PDLCs were stimulated with *E. coli* LPS (1, 5, and 10 μg/mL for 6 h and 12 h). GCF were collected from periodontitis patients and healthy volunteers.	LPS suppressed PDLCs viability and led to production and secretion of IL-1β, IL-18, IL-6, and TNF-α in a time- and concentration-dependent manner. LPS activated NLRP3 and GSDMD, cleaved caspase-1, and upregulated GSK-3β. Blockage of GSK-3β restrained NLRP3-mediated pyroptosis. Pro-inflammatory cytokines were upregulated in periodontal patients' GCF but not in healthy volunteers.	Glycogen synthase kinase-3β (GSK-3β) deficiency inactivates the NLRP3 inflammasome-mediated cell pyroptosis in LPS-treated periodontal ligament cells (PDLCs) [4]
2	Oka S, Li X, Sato F, Zhang F, Tewari N, Kim I-S, Zhong L, Hamada N, et al.	2021	Journal of Periodontal Research	In vitro and in vivo	HGFs and PDLCs were stimulated with *P. gingivalis* LPS (10 μg/mL for 24 h). Mouse experimental periodontitis model (WT and differentiated embryo chondrocyte 2 (Dec2) KO) was established.	LPS activated caspase-1, caspase-11, and NF-κB. Dec2 KO upregulated LPS-induced pyroptosis, resulting in IL-1β release. The inhibition of Dec2 led to the activation of caspase-1 and GSDMD, reduced the phosphorylation and translocation of NF-κB, decreased IL-1β expression, reducing pyroptosis.	A deficiency of Dec2 triggers periodontal inflammation and pyroptosis [1]
3	Chen Q, Cao M, Ge H.	2021	BioMed Research International	In Vitro	PDLCs were treated with *P. gingivalis* LPS (100 ng/mL for 72 h). The expression of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and miR-769-5p in gingival tissues of patients with periodontitis and LPS-treated PDLCs was evaluated.	MALAT1 KO promoted cell viability and inhibited inflammation and pyroptosis. The expression of MALAT1 and hypoxia-inducible factor 3A (HIF3A) was enhanced, and the expression of miR-769-5p was reduced in gingival tissues of patients with periodontitis and LPS-treated PDLCs.	Knockdown of MALAT1 inhibits the progression of chronic periodontitis via targeting miR-769-5p/HIF3A axis [13]

Table 1. *Cont.*

	Authors	Year	Journal	Study Type	Methods	Outcomes	Title and Reference
4	Liu S, Du J, Li D, Yang P, Kou Y, Li C, Zhou Q, Lu Y, et al.	2020	Journal of Molecular Histology	In Vitro	Human osteoblast-like cells were exposed to *E. coli* LPS (0.5, 1, or 2 μg/mL) for 24 h and 48 h. *N*-acetyl-*L*-cysteine (NAC) was used to decrease the intracellular ROS level and MCC950 was used to inhibit pyroptosis.	LPS led to NLRP3-mediated pyroptosis in a time- and dose-dependent manner. The inhibition of ROS with NAC attenuated oxidative stress-mediated pyroptosis. The inhibition of pyroptosis with MCC950 restored the expression of osteogenic differentiation-related proteins of osteoblasts.	Oxidative stress induced pyroptosis leads to osteogenic dysfunction of MG63 cells [10]
5	Cheng R, Feng Y, Zhang R, Liu W, Lei L, Hu T.	2018	Biochimica et Biophysica Acta—Molecular Basis of Disease	In vitro and in vivo	PDLCs were stimulated with *E. coli* LPS (1 μg/mL) or *P. gingivalis* LPS (10 μg/mL) for 24 h. Rat experimental periodontitis model was established. VX765 caspase-1 inhibitor was used to block pyroptosis.	VX765 inhibited the expressions of IL-1β, monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-8 In Vitro, decreasing inflammatory responses during periodontitis. VX765 suppressed bone loss in vivo, linking pyroptosis to bone resorption in acute apical periodontitis.	The extent of pyroptosis varies in different stages of apical periodontitis [3]
6	Chen R, Liu W, Zhang R, Feng Y, Bhowmick NA, Hu T.	2017	Frontiers in Cellular and Infection Microbiology	In vitro and in vivo	HGFs were stimulated with *E. coli* LPS (1 μg/mL) or *P. gingivalis* LPS (10 μg/mL) at 2% or 20% O_2 for 6 h. Mouse experimental periodontitis model was established.	*P. gingivalis* LPS slightly decreased the level of NLRP3 and IL-1β under normoxia. Hypoxia reversed the effects of *P. gingivalis* LPS, promoting caspase-1 activation and IL-1β maturation. *E. coli* LPS enhanced IL-1β maturation in both normoxia and hypoxia, and turned normoxia into hypoxia in the periodontitis model, suggesting to increase the inflammatory effect of *P. gingivalis* LPS.	*Porphyromonas gingivalis*-derived lipopolysaccharide combines hypoxia to induce caspase-1 activation in periodontitis [14]

Table 1. *Cont.*

	Authors	Year	Journal	Study Type	Methods	Outcomes	Title and Reference
7	Cecil JD, O'Brien-Simpson NM, Lenzo JC, Holden JA, Singleton W, Perez-Gonzalez A, Mansell A, Reynolds EC.	2017	Frontiers in Immunology	In vitro and in vivo	THP-1 (monocytes) and macrophages extracted from C57BL/6 J mice (ex vivo and in vivo) were treated with intraperitoneal injections of *P. gingivalis*, *T. denticola*, and *T. forsythia* OMVs (100 ng protein/mL) for 4 h. Cells were stimulated with nigericin (10 µM), silica (125 mg/mL), or transfected with poly(dAdT) (250 ng/mL) using lipofectamine LTX for 6 h.	OMVs interacted with monocytes and macrophages, inducing phagocytosis, NF-κB activation, IL-1β secretion, and cell death via NLRP3 activation. The immune stimulatory effects of *P. gingivalis* OMVs are suggested to dysregulate the host immune response and initiate the disease, while the pro-inflammatory effects of *T. denticola* and *T. forsythia* OMVs are suggested to promote disease progression.	Outer membrane vesicles prime and activate macrophage inflammasomes and cytokine secretion In Vitro and in vivo [15]
8	Fleetwood AJ, Lee MKS, Singleton W, Achutan A, Lee M-C, O'Brien-Simpson NM, Cook AD, Murphy AJ, et al.	2017	Frontiers in Cellular and Infection Microbiology	In vitro and in vivo	C57BL/6 mouse and human macrophages were treated with viable *P. gingivalis*, heat-killed *P. gingivalis*, OMVs, or heat-inactivated OMVs at a MOI of 10:1, 25:1 (protein concentration of about 3.0 µg/mL) or 100:1 bacilli or OMVs/cell for 2 h.	*P. gingivalis* did not lead to the activation of NLRP3 while *P. gingivalis* OMVs activated caspase-1, produced large amounts of IL-1β and IL-18, released lactate dehydrogenase (LDH), and were positive for 7-amino actinomycin D (7-AAD) staining, thus indicating of pyroptosis.	Metabolic remodeling, inflammasome activation, and pyroptosis in macrophages stimulated by *Porphyromonas gingivalis* and its outer membrane vesicles [16]
9	Lu WL, Song DZ, Yue JL, Wang TT, Zhou XD, Zhang P, Zhang L, Huang DM.	2017	International Endodontic Journal	In Vitro	PDLCs were stimulated with MDP (10 µg/mL) for 0, 1, 3, 8, 14 or 24 h; *E. coli* LPS (0.5 µg/mL) for 0, 4, 8 or 24 h; or MDP and LPS in combination for 0, 4, 8 or 24 h.	MDP, LPS, or MDP in combination with LPS promoted the expression of NLRP3, caspase-1, and induced IL-1β secretion. MDP exhibited synergistic or additive effects with LPS to upregulate the expression of NLRP3, ASC and caspase-1.	NLRP3 inflammasome may regulate inflammatory response of human periodontal ligament fibroblasts in an apoptosis-associated speck-like protein containing a CARD (ASC)-dependent manner [6]

Table 1. *Cont.*

	Authors	Year	Journal	Study Type	Methods	Outcomes	Title and Reference
10	Brown PM, Kennedy DJ, Morton RE, Febbraio M.	2015	PLoS ONE	In Vivo	Cd36/Ldlr and Ldlr mice were derived from a cross between Cd36° and Ldlr mice. *P. gingivalis* (~2 × 10^9 CFU/mL) were resuspended in saline containing 2% carboxymethylcellulose (as a thickener to promote adherence) prior to oral inoculation of mice.	An increase of 225% (females) and 175% (males) was found in periodontal lesions compared to uninfected mice. This increase was CD36/SR-B2-dependent since there was no significant change in lesion burden between infected and uninfected Cd36°/Ldlr mice. Activation of the NLRP3 by *P. gingivalis* is mediated by CD36/SR-B2 and TLR2, leading to systemic release of IL-1β and inducing pyroptosis.	CD36/SR-B2-TLR2 dependent pathways enhance *Porphyromonas gingivalis* mediated atherosclerosis in the Ldlr KO mouse model [17]
11	Taxman DJ, Swanson KV, Broglie PM, Wen H, Holley-Guthrie E, Huang MT-H, Callaway JB, Eitas TK, et al.	2012	Journal of Biological Chemistry	In vitro and in vivo	*MyD88*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ mice macrophages were infected with *P. gingivalis*. Macrophages were stimulated with *E. coli* LPS (1 µg/mL) for 3 h, followed by ATP (2 mM) for 0.5 h, nigericin (20 µM) for 0.5 h, monosodium urate (200 µg/mL) for 6 h, alum crystals (400 µg/mL) for 6 h, or *S. aureus* peptidoglycan (20 µg/mL) for 14-16 h.	*P. gingivalis* lacks signaling capability for the NLRP3 activation and can suppress NLRP3 activation by *F. nucleatum*, thus repressing IL-1β and IL-18 release and cell death. *P. gingivalis* can repress NLRP3 activation by *E. coli*, and by DAMPs and PAMPs that mediate activation through endocytosis, but cannot suppress NLRP3 activation by ATP or nigericin, suggesting that *P. gingivalis* preferentially suppress endocytic pathways towards NLRP3 activation.	*Porphyromonas gingivalis* mediates inflammasome repression in polymicrobial cultures through a novel mechanism involving reduced endocytosis [18]

Table 1. *Cont.*

	Authors	Year	Journal	Study Type	Methods	Outcomes	Title and Reference
12	Domon H, Takahashi N, Honda T, Nakajima T, Tabeta K, Abiko Y, Yamazaki K.	2009	Clinica Chimica Acta	In Vitro	Cells were obtained from human periodontitis patients. Macrophages were stimulated with *E. coli* LPS (1 μg/mL), *P. gingivalis* LPS (1 μg/mL), IFN-γ (100 or 500 U), or tunicamycin (1 μg/mL) for 1, 3, 6, 12, or 24 h. The expression of unfolded protein response (UPR) was analysed.	The expression of UPR-related genes was higher in periodontitis than in gingivitis lesions. *P. gingivalis* LPS (but not *E. coli* LPS or IFN-γ) failed to up-regulate gene expressions. Macrophages stimulated with *E. coli* LPS or IFN-γ expressed IL-β and caspase-4 at the gene level while tunicamycin did not.	Up-regulation of the endoplasmic reticulum stress-response in periodontal disease [7]

Abbreviations: Colony forming units (CFU); Gasdermin D (GSDMD); Gingival crevicular fluid (GCF); Glycogen synthase kinase-3β (GSK-3β); Human gingival fibroblasts (HGFs); Interleukin (IL); Knockout (KO); Lipopolysaccharides (LPS); Multiplicity of infection (MOI); Nuclear factor kappa B (NF-kB); Outer membrane vesicles (OMVs); Pathogen-associated molecular patterns (PAMPs); Periodontal ligament (PDL); Primary human periodontal ligament cells (PDLCs); Reactive oxygen species (ROS); Wild-type (WT).

Table 2. Literature review article.

Authors	Year	Journal	Main Findings	Title and Reference
De Andrade KQ, Almeida-da-Silva CLC, Coutinho-Silva R.	2017	Mediators of Inflammation	Inflammasomes are involved in the pathogenesis of periodontitis; however, it is necessary to determine which inflammasomes, others than the typical NLRP3, contribute to the pathogenesis of periodontitis induced by *P. gingivalis* and *F. nucleatum*. With more solid literature on the signaling pathways and immune responses during infection with these bacteria, more effective treatments for periodontitis may appear.	Immunological pathways triggered by *Porphyromonas gingivalis* and *Fusobacterium nucleatum*: therapeutic possibilities? [8]

Table 3. Excluded articles.

	Authors	Year	Journal	Type of Study	Reason of Exclusion	Title and Reference
1	Wang J, Du, C, Xu L.	2021	Archives of Oral Biology	In Vitro	Studied apoptosis	Circ_0081572 inhibits the progression of periodontitis through regulating the miR-378h/RorA axis [19]
2	Liu P, Cui, L, Shen L.	2020	Bioscience Reports	In Vitro	Studied apoptosis	Knockdown of TRIM52 alleviates LPS-induced inflammatory injury in human periodontal ligament cells through the TLR4/NF-κB pathway [20]
3	Zhang K, He S, Dai Z, Cao L, Yue S, Bai Y, Zheng M.	2020	Archives of Oral Biology	In Vitro	Studied apoptosis	Axin 1 knockdown inhibits osteoblastic apoptosis induced by Porphyromonas gingivalis lipopolysaccharide [21]
4	Zhou Y, Zhang H, Zhang G, He Y, Zhang P, Sun Z, Gao Y, Tan Y.	2018	Molecular Medicine Reports	In Vitro	Studied apoptosis	Calcitonin gene-related peptide reduces Porphyromonas gingivalis LPS-induced TNF-α release and apoptosis in osteoblasts [22]
5	Shirasugi M, Nishioka K, Yamamoto T, Nakaya T, Kanamura N.	2017	Biochemical and Biophysical Research Communications	In Vitro	Studied apoptosis and cytostasis	Normal human gingival fibroblasts undergo cytostasis and apoptosis after long-term exposure to butyric acid [23]
6	Zhu X, Lu W, Chen Y, Cheng X, Qiu J, Xu Y, Sun Y.	2016	PLoS ONE	In Vitro	Studied apoptosis	Effects of *Porphyromonas gingivalis* Lipopolysaccharide olerized monocytes on inflammatory responses in neutrophils [24]
7	Deepak V, Kasonga A, Kruger MC, Coetzee M.	2016	Biological and Pharmaceutical Bulletin	In Vitro	Studied apoptosis	Carvacrol inhibits osteoclastogenesis and negatively regulates the survival of mature osteoclasts [25]
8	Jönsson D, Nilsson B-O.	2012	Journal of Periodontal Research	In Vitro	Studied apoptosis	The antimicrobial peptide LL-37 is anti-inflammatory and proapoptotic in human periodontal ligament cells [26]
9	Zaric, S, Shelburne C, Darveau R, Quinn DJ, Weldon S, Taggart CC, Coulter WA.	2010	Infection and Immunity	In Vitro	Studied apoptosis	Impaired immune tolerance to *Porphyromonas gingivalis* lipopolysaccharide promotes neutrophil migration and decreased apoptosis [27]
10	Thammasitboon K, Goldring SR, Boch JA.	2006	Bone	In Vitro	Studied apoptosis	Role of macrophages in LPS-induced osteoblast and PDL cell apoptosis [28]

4. Discussion

The results of this review are divided. Part 1 considers the fundamental concepts and related factors of pyroptosis. Part 2 discusses the results found in Tables 1 and 2, highlights the clinical relevance of pyroptosis on periodontal diseases, and considers pyroptosis in periodontal therapy.

4.1. Part 1—Updated Knowledge of Pyroptosis Inflammatory Pathways

4.1.1. Innate Immune System and Inflammation

Innate immune responses are tightly regulated by various pathways to control infections and maintain homeostasis [29]. Hence, the innate immune system is strongly related to inflammation, which is a host tissue response to an assault commonly triggered by microorganisms or other stimuli such as chemicals, radiation, or trauma, and their released products (i.e., metabolites, endotoxins). Consequently, inflammation is generally related to pathologies, while it is important to highlight that inflammatory reactions provide rapid and early protection against potential aggressor agents. Clinically, host tissues experience several inoffensive inflammatory reactions routinely, as a result of various stimuli, and these responses are not considered infections or diseases. These cases represent situations where the inflammatory response is physiologic and provide early protection from potentially dangerous events [30]. Nevertheless, when the innate immune system is acting against pathogens or other potential dangers, the inflammatory responses are initiated through pattern recognition receptors, phagocytes, dendritic cells, epithelial cells that recognise pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) [31–33]. These stimulate the transcription level of interferons, pro-inflammatory cytokines, interleukins, and other essential factors, further leading to the recruitment of other immune cells (such as lymphocytes) to bridge innate and adaptive immune systems [33].

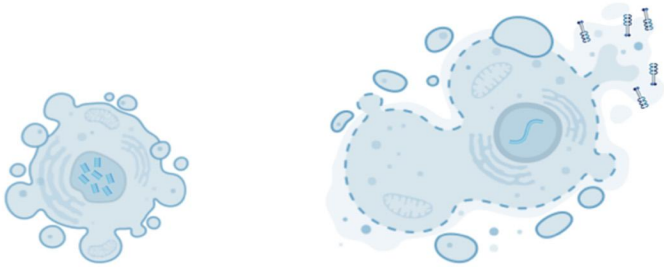
The inflammasome is one of the pathways of the innate immune system, which activates a family of cysteine proteases called inflammatory caspases. These caspases control the immune response by cleaving specific cellular substrates [9,29,34]. Inflammasomes often require activation by danger signals. The inflammasome activation can lead to the release of pro-inflammatory cytokines and, more interestingly, to an inflammatory programmed cell death known as pyroptosis [31,33].

4.1.2. Pyroptosis

Cell death can be categorised by describing the initiating events, the intermediate changes, the terminal cellular events, and its effect on tissue. Each cell death pathway may be distinguished based on these four categories [35]. Apoptosis was the first well-recognised type of cell death, and this "programmed cell death" is broadly applied to several endogenous genetically defined pathways in which the cell plays an active part in its own destruction. However, other cell death paths include autophagy [36], oncosis [37], necroptosis [38,39], NET (neutrophil extracellular traps)osis [40], ferroptosis [41], cytostasis [23], pyroptosis [42,43], among others yet to be discovered. Most of these modalities have specific initiation events In vitro, but not all have well-defined roles in vivo [35].

Pyroptosis is as an efficient mechanism of bacterial clearance developed by the innate immune system [34]. It was first described in 1992 in macrophages infected with *Shigella flexneri* [44] and later when a similar phenotype was observed after infection with *Salmonella typhimurium* [34,45,46]. Pyroptosis is a process of cellular self-destruction mediated by caspases and, therefore, it was not initially distinguished from the classic apoptosis. However, the mechanisms, characteristics, and outcomes of pyroptosis are very distinct from apoptosis, where the most significant difference is the inflammatory responses (Figure 2) [9,16,34,35,42,47]. Additionally, pyroptosis occurs rapidly, and it is accompanied by the release of numerous pro-inflammatory factors [5]. Thus, the term pyroptosis (from the Greek 'pyro', relating to fire or fever, and 'ptosis', denoting a falling) is used to describe

the remarkable pro-inflammatory process of cell death on pyroptosis [46]. As a clinical example, pyroptosis of peripheral blood mononuclear cells was associated with the severity and the mortality of patients with sepsis [48].



	Apoptosis	Pyroptosis
Membrane	Integrity	Disruption (pores)
Nuclei	DNA fragmentation	Swelling
Inflammasome activation	Caspases-3/-8/-9	Caspases-1/-4/-5 (humans) and -11 (mice)
Cytokines	—	IL-1 and IL-18
Inflammation	Rare	Pro-inflammatory

Figure 2. Comparison between apoptosis and pyroptosis. Based on Liu et al., 2018 [47].

Caspase-1 was first recognised as a protease that processes the inactive precursors of interleukins-1β (IL-1β) and -18 (IL-18) into mature inflammatory cytokines [7,49]. However, caspase-1 activation can result not only in the production of activated inflammatory cytokines but also in rapid cell death characterised by plasma-membrane rupture and release of pro-inflammatory intracellular contents [42,43]. Additionally, DNA cleavage during pyroptosis results from the activity of an unidentified activated caspase-1 nuclease that does not produce the oligonucleosomal DNA fragmentation pattern that is characteristic of apoptosis. DNA cleavage is accompanied by marked nuclear condensation while nuclear integrity is maintained [42,43,50]. Pyroptosis also presents a series of morphological and physiological changes related to the inflammatory response [9,34,51]. Morphologically, small pores (10–15 nm) emerge on the membrane of pyroptotic cells, which change the membrane's permeability [39]. This event contributes to intracellular bacterial clearance and destroys any niche formed by intracellular bacterial replication because it causes intracellular bacterial exposure to the extracellular compartment, making bacteria more susceptible to antibodies and attacks by phagocytes such as neutrophils [8,9,34]. Additionally, numerous pro-inflammatory cytokines in the cytoplasm are released from the pores to the extracellular matrix [52], promoting cell lysis and death. Finally, the cells burst and an inflammatory response around the dead cells is triggered because of the released cytokines [53]. In contrast, apoptosis involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells [47].

When the cell is stimulated, PAMPs and DAMPs promote the formation of inflammasomes. Pyroptosis is mediated by the activation of caspase-1 by the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing protein 3 (NLRP3) inflammasome [5,6]. Caspase-1 thus cleaves the members of the gasdermin family, including gasdermin D (GSDMD), which subsequently results in the perforation of the cell membrane due to the release of its N-terminal domain [5]. This caspase-1 triggering through NLRP3 is the classical canonical inflammasome activation of pyroptosis, while the non-canonical activation of pyroptosis happens through the triggering of caspases-4 and -5 in humans, or caspase-11

in mice [7–9]. Both pathways lead to the cleavage of GSDMD. Therefore, the mechanisms of pyroptosis basically have to involve the downstream of GSDMD, finally leading to pores on the cell membrane, while cytoplasmic molecules, such as IL-1β and IL-18, are released from the pores and provoke a robust inflammatory response (Figure 1) [2,9,10]. Hence, the manifestation of pyroptosis can be determined by a combination of markers, including the activation of caspases-1, -4, -5, and -11, the cleavage of GSDMD, and the activation and release of interleukins IL-1β and IL-18 [9]. Finally, how the pyroptosis pathway is activated is explained by the virulence factors.

4.1.3. Virulence Factors

Healthy cells do not release interleukins when the cells are dying. However, certain virulence factors may activate the inflammasome pathway, leading to cell death and inflammation of the surrounding tissues [54,55]. Different types of classical virulence factors may act on the activation of distinct caspases that will determine the type of cell death.

Staphylococcal α-hemolysin is a bacterial pore-forming toxin produced by *Staphylococcus aureus*, which activates inflammasome activity and caspase-1, thus inducing pyroptosis [56]. *S. aureus* exploits the pro-inflammatory bias of human keratinocytes to activate pyroptosis, which is required for staphylococci to penetrate across the cell membrane [57]. The α-hemolysin role in the pathogenesis of skin infection is well documented [58–60]. Still, it remains unclear exactly how these non-motile bacteria invade through the barrier posed by the multiple layers of proliferating and cornified keratinocytes that comprise normal human skin [57].

Nigericin is a microbial toxin produced by *Streptomyces hygroscopicus* that decreases the intracellular potassium (K^+), which causes caspase-1 activation, leading to pyroptosis [61]. Nigericin binds to K^+, which is subsequently transported across the plasma membrane as nigericin-K and released on the outside of the cell [62]. Nigericin has been shown to activate NLRP3 inflammasome and induce the release of IL-1β [63,64].

Lipopolysaccharides (LPS) are toll-like receptor (TLR) agonists that are found in the outer membrane of Gram-negative bacteria [30]. LPS have a pro-inflammatory function via modulation of caspases that can cleave GSDMD, the pro-pyroptotic factor (Figure 1) [1,65,66]. Most of the oral pathogens are Gram-negative bacteria, such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Fusobacterium nucleatum*, *Tannerella forsythia*; therefore, these pathogens are all able to produce the virulence factor LPS [2,3,5,8,14,15,18,67,68].

4.1.4. Inflammasomes

Inflammasomes are cytosolic multi-protein complexes that perform inflammatory responses when stimulated by pathogens or endogenous hazards [33,69,70]. There are two main classes of inflammasome sensor proteins: (1) nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing proteins (Noll Like Receptors or NLR) and (2) absent in melanoma 2 (AIM2)-like receptors [32]. The oligomerisation of NLR and AIM2-like receptor sensors facilitates the oligomerisation of adapters such as apoptosis-associated, speck-like protein containing a caspase recruitment domain (ASC) [71]. These adaptors trigger the recruitment of effectors, such as pro-caspase-1, that are activated and cleaved into their mature forms [32,33]. Thus, inflammasomes are of central importance to inflammatory processes, as they promote the cleavage of pro-inflammatory cytokines, notably IL-1β and IL-18, through the maturation of caspase-1 [70,72]. Dysregulations or gene mutations of inflammasomes are associated with several auto-inflammatory diseases and cancer [33,73].

Regarding pyroptosis, the NLRP3 inflammasome seems to be the one to act on the activation of caspase-1. Thus, caspase-1 is the essential mediator of inflammasome function and its activity is a direct marker of NLRP3 activation [6,72]. Emerging evidence suggests that the NLRP3 inflammasome can react to a wide range of bacterial ligands, including LPS, bacterial RNA, and peptidoglycans (PAMPs or DAMPs), and plays a pivotal role in

the pathogenesis of several diseases, such as rheumatoid arthritis, bone loss, osteomyelitis, periodontal disease, and others, by regulating the inflammatory response. Overexpression of NLRP3 exacerbates inflammatory osteolysis and inhibits calcium deposition in metabolic bone diseases [5,33,69,74]. In this sense, caspases have a close relationship with inflammasomes, once their activity triggers the caspase activation.

4.1.5. Caspases

Caspase is an abbreviation for Cysteine-dependent ASPartate-specific proteASE or cysteinyl aspartate specific proteinase [9]. Caspase-1 is the leading enzyme to mediate the highly inflammatory process known as pyroptosis, which is characterised by rapid cell lysis and the release of pro-inflammatory cytokines [73]. The downstream processes, resulting from caspase-1 activation, are dictated by the cell type and the nature and magnitude of the stimulus received [34,42]. Thus, caspase-1 activation is a host defence mechanism. Pathogens require mechanisms to prevent the potent inflammatory outcome of pyroptosis to persist and cause disease. Likewise, the host should possess means to neutralise pathogen-mediated regulation of caspase-1 activity and successfully control the infection [42]. Nevertheless, although pyroptosis has this protective host response to infectious diseases, exaggerated caspase-1 activation can be detrimental to the surrounding tissues [42].

Caspase-1 is pivotal for pyroptosis. It was originally termed "interleukin converting enzyme" for its well-established role in the cleavage of IL-1β and IL-18 [29]. Upon sensing PAMPs and DAMPs, innate immune cells form inflammasomes that recruit and activate caspase-1, known as the canonical inflammasome pathway. Other inflammatory caspases, such as caspase-4 and -5, directly bind bacterial LPS, triggering pyroptosis, which is the non-canonical inflammasome pathway. However, the non-canonical pathway ultimately leads to canonical inflammasome engagement through caspase-1 activation (Figure 1) [29]. By including specific caspase-1 inhibitors—-Ac-YVAD-CHO, for instance, it is possible to discriminate caspase-1 activity from the activity of other caspases, and pyroptosis from other types of cell death [72]. In addition, the activated caspase-1 has a critical role in the cleavage of the GSDMD, another central element of pyroptosis (Figure 1).

4.1.6. Gasdermin D

The GSDM family includes GSDM A, B, C, D, and E, as well as DFNB 59 [75], of which GSDMD is the most important mediator of pyroptosis. GSDMD is cleaved by caspase-1 into two fragments: (1) the N-terminal fragment, and its inhibitory counterpart, (2) the C-terminal fragment. The N-terminal domain can form small pores of 10–15 nm on the cell membrane, that allow the secretion of the cytoplasmatic content, including invading pathogens and pro-inflammatory cytokines. Such cytokines recruit more inflammatory cells to trigger the inflammatory cascade. Additionally, GSDMD pores generate potassium efflux that allow caspase-1 activation through NLRP3 inflammasome (Figure 1) [1,9,29,47]. Thus, GSDMD is a central effector of pyroptosis that has different roles inside the cells, while the most remarkable activity is the formation of pores in the cell membrane, which allow the release of interleukins to the extracellular matrix, then provoking an intense inflammatory reaction.

4.1.7. Interleukins

Pyroptosis is predicted to be pro-inflammatory due to the release of inflammatory cytokines [34]. The cytokines related to pyroptosis are the interleukins IL-1β and IL-18. IL-1β is a potent endogenous pyrogen that stimulates vasodilation, fever, leukocyte tissue migration, immune cell extravasation, and expression of several cytokines and chemokines [34,76]. Macrophages are a prime source of pro-IL-1β that generally depend on caspase-1 for maturation and secretion of the biologically active IL-1β [77]. IL-18 promotes interferon-γ production and activates T cells and macrophages [34,78]. Both IL-1β and

IL-18 play crucial parts in the pathogenesis of a range of inflammatory and autoimmune diseases [18,76,78].

The ligation of pattern recognition receptors by PAMPs leads to intracellular production of pro-IL-1β and pro-IL-18. Simultaneous ligation of receptors for DAMPs leads to assembly of NLRP3 and cleavage of pro-caspase-1 into activated caspase-1, which will finally cleave pro-IL-1β and -18 into their mature forms [2,79]. Infections, by many types of intracellular bacteria, stimulate the synthesis of pro-IL-1β, but not its secretion. It has been shown that a second signal, often due to a danger signal (DAMPs) such as extracellular ATP or nigericin, is then able to activate NLRP3 and caspase-1 [77,79]. Hence, pyroptosis is a way to release the processed IL-1β and IL-18 from the cell. Nevertheless, depending on the cell type and stimulus, inflammasome engagement, and caspase-1 activation, IL-1β release may occur in the absence of cell death. Although no cytokines are required for cell death, their production contributes to the inflammatory response generated by cells under pyroptosis [9,42]. The clinical consequences are exacerbated inflammatory reaction, tissue damage, and disease.

4.1.8. Clinical Relevance

Inflammasome mutations can lead to inappropriate caspase-1 activation, which is associated with autoinflammatory syndromes [80]. Moreover, caspase-1 is involved in the pathogenesis of several diseases, including periodontal disease [4], Alzheimer's disease [81,82], cardiovascular disease [83], rheumatoid arthritis [84], endometriosis [70], and Crohn's disease [85], all of which are characterised by cell death and inflammation. Caspase-1 deficiency or inhibition protects against cell death, inflammation, and tissue dysfunction, associated with these diseases. Thus, caspase-1 is a potential therapeutic target through specific pharmacological inhibitors [29,42]. However, it is essential to emphasise that caspase-1 is also part of the immune system and thus crucial for protection against virulence factors. Research to identify and characterise novel caspase substrates can also expand the understanding of inflammatory caspases in health and disease. Consequently, research should address how endogenous mechanisms and inhibitors control inflammatory caspase activity. Pyroptosis and other caspase-1-dependent processes are therefore relevant to the understanding of beneficial host responses and medical conditions for which inflammation is central to the pathophysiology of the disease [42].

Considering that pyroptosis is strongly associated with inflammatory diseases and that the virulence factors existing in the oral environment can provoke the exacerbation of the pyroptosis towards strong inflammation and tissue damage, it is likely that pyroptosis is associated with periodontal disease. In the meantime, such inflammasome pathway on the periodontal tissue is poorly explored. Hence, Part 2 of this discussion will argue the role of pyroptosis on periodontal disease.

4.2. Part 2—Pyroptosis on the Periodontal Diseases and Periodontal Therapy

The knowledge of pyroptosis in the pathogenesis of periodontitis is evolving. This can be noticed from the articles retrieved from the search strategy applied herein. It is also clear that other areas of medical knowledge besides dentistry focus on understanding pyroptosis-mediated inflammation processes [12,75,85–90]. Regarding periodontitis and periodontal therapies, the included articles present similarities regarding methods, type of cells involved, concentration, and kind of pyroptosis stimulation, as the outcomes point to the same direction considering the cellular events. Therefore, the gathered data led to a discussion on the related processes and the clinical relevance of studying pyroptosis in periodontitis.

Periodontal disease is one of the most prevalent infectious human inflammatory diseases, and it is characterised by the inflammatory reaction and the progressive destruction of the tooth-supporting tissues [30]. It is a response to years of prolonged exposure to a polymicrobial community in the gingiva and periodontal pocket [30], as shown in Figure 3. Periodontitis is associated with Gram-negative anaerobic bacteria, such as *P. gingivalis*,

A. actinomycetemcomitans, *T. denticola*, *F. nucleatum*, *T. forsythia*, among others found in the dental biofilms [2,3,5,8,14,15,18,67,68]. Gram-negative bacteria are specialised in the production of virulence factors that can trigger periodontal disease. Virulence factors are critical in manipulating and exploiting host immune responses, leading to dysbiosis in the oral cavity and periodontitis progression [54].

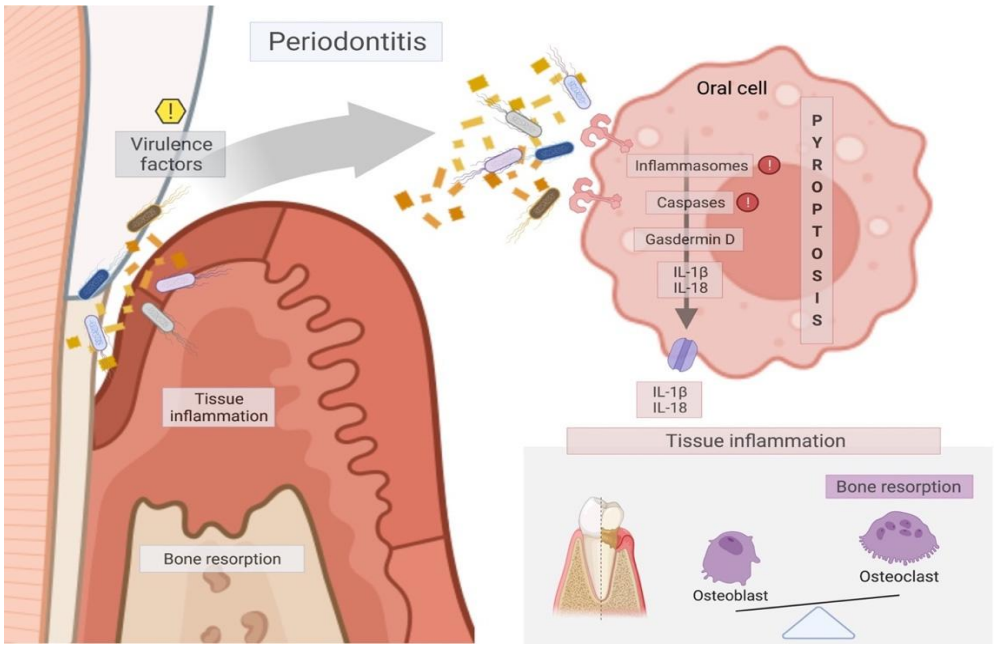


Figure 3. Schematic diagram of the mechanism of pyroptosis pathway on periodontal tissues to the promotion of periodontitis. Virulence factors activate the inflammasome/caspase downstream to the cleavage of Gasdermin D, which is responsible for membrane pore formation and, thus, the release of interleukins IL-1β and IL-18 to the extracellular environment. Those interleukins lead to tissue inflammation and disruption of the balance between bone formation by the osteoblasts and bone resorption by the osteoclasts, thus aggravating the process of periodontitis through soft tissue inflammation (swelling, bleeding) and marginal bone loss. Red exclamation marks mean the main targets for pyroptosis-specific inhibitors, such as MCC950, Ac-YVAD-CHO, Z-LEVD-FMK, and VX765. Yellow exclamation mark indicates the main target for therapeutic approaches that should act on the virulence factors responsible for triggering pyroptosis on periodontal tissues.

4.2.1. Clinical and In Vivo Pieces of Evidence of Pyroptosis on the Periodontal Tissues

The team of Bostanci and Belibasakis reported that inflammasomes in gingival tissues were significantly higher in patients with periodontal disease than healthy patients [55]. Immunohistochemistry confirmed, particularly in the periodontal epithelium layer, that the overall intensity of NLRP3 expression was higher in chronic periodontitis and patients with generalised aggressive periodontitis compared to healthy control subjects [91]. Consistently, NLRP3, caspase-1, caspase-4, and IL-18 was more pronounced in the inflammatory gingiva compared to healthy gingiva [92], similar to what was observed in a rat model exposed to *P. gingivalis* LPS, where caspase-11 was also raised [92]. In addition, the removal of *P. gingivalis* from subgingival biofilms led to the restored expression of NLRP3 and IL-1β [55]. In other models, pyroptosis markers, such as GSDMD [1], NLRP3 [3,14], cleaved caspase-1 [3,14], and IL-1β [1,3,14] were upregulated in diseased periodontal tissues compared to healthy controls. Pyroptosis seems to have an impact on alveolar bone too. Loss-of-function of caspase-1 but not of NLRP3 reduced *A. actinomycetemcomitans*-induced bone resorption in mice [69], implying that caspase-1 is instrumental in modulating inflammation

caused by the pathogen [5]. Taken together, there is evidence for pyroptosis signaling in inflamed periodontal tissues, and caspase-1 partially mediates inflammatory osteolysis.

4.2.2. In Vitro Research on Pyroptosis on the Periodontal Disease

Studies collected from the search strategy applied herein (Table 1) point to similarities regarding In vitro analyses, especially regarding the induction of pyroptosis via LPS from both *E. coli* and *P. gingivalis*. *E. coli* LPS led to IL-1β and IL-18 secretion, activated NLRP3 and GSDMD, and cleaved caspase-1 in PDLCs [4], which agrees with previous findings of *E. coli* LPS-stimulated PDLCs, leading to the expression of NLRP3 and caspase-1 and IL-1β secretion [6]. Another study found that *P. gingivalis* LPS activated caspase-1 and caspase-11 in HGFs and PDLCs [1]. Likewise, HGFs stimulated with *P. gingivalis* LPS under hypoxia promoted caspase-1 activation and IL-1β maturation, while *E. coli* LPS also enhanced IL-1β maturation under normoxia [14]. Other In vitro research suggests that hypoxia can be used as an activation signal together with a "startup signal" of LPS to complete the entire pyroptosis pathway [14,74]. Furthermore, macrophages obtained from periodontitis patients were stimulated with *E. coli* LPS and *P. gingivalis* LPS while the expression of caspase-4 and IL-1β was seen for the cells stimulated with *E. coli* LPS [7]. Hence, it seems that *E. coli* LPS has stronger effects on pyroptosis or even potentialise *P. gingivalis* LPS effects in vitro. Alternatively, *P. gingivalis* induced pyroptosis of HGFs by activation of caspase-1 and NLRP6 [47]. Additionally, HGFs infected with *T. denticola* activated caspase-4 and released IL-1β [93], and LPS increased caspase-1 and NLRP3 in mesenchymal cells isolated from the umbilical cord [94]. The In vitro studies presented herein focused on LPS and how it affects mesenchymal cells and macrophages (Table 1). There is, however, a lack of evidence on how other virulence factors than LPS affect pyroptosis and how this affects other cell types, such as epithelial cells.

4.2.3. Virulence Factors Associated with Pyroptosis on the Periodontal Disease

Virulence factors impair the epithelial barrier functions and thus allow the bacterial invasion of the gingiva [95]. Virulence factors also support the dissemination of the bacteria via the bloodstream into peripheral tissues [96] and then assist the bacterium to colonize the new environment [97]. Even though most studies comprise LPS, other virulence factors produced by periodontopathogens are outer membrane vesicles (OMVs), fimbriae, capsules, gingipains, and leukotoxin (LtxA), among many others. They all have roles in regulating immune responses during periodontitis progression [54,98].

OMVs produced by *P. gingivalis* can penetrate host tissues and interact with monocytes and macrophages, inducing strong pro-inflammatory responses, IL-1β secretion, and inflammatory cell death via inflammasome activation [15,16]. Periodontal OMVs produced by *A. actinomycetemcomitans* were internalised into the perinuclear region of HGFs and triggered the innate immunity via carriage of NOD1- and NOD2-active PAMPs [99]. Proteomics of OMVs by *A. actinomycetemcomitans* affirmed the role of such OMVs in periodontal and systemic diseases [100]. *E. coli* OMVs act as a delivery system for cytosolic LPS, which binds and activates cytosolic caspases-11, -4, and -5 to trigger caspase-1-independent pyroptosis through the cleavage of the pore-forming GSDMD [101]. Additionally, OMVs from *T. denticola* and *T. forsythia* can promote disease progression [15].

Fimbriae and capsules can adhere to other bacteria, host tissues, and cells to promote biofilm formation [54]. *E. coli* fimbriae increased IL-1β release from neutrophils involving caspase-1 and NLRP3 activation and stimulated the antimicrobial activity of human neutrophils against *E. coli* [102].

Gingipains provide *P. gingivalis* with the ability to evade host immune responses and clearance, especially through the degradation of extracellular matrix components. *P. gingivalis* strains KDP136 (gingipain-null mutant) or KDP150 (FimA-deficient mutant) are also less pathogenic with respect to NLRP3 activation compared to the original WT strains [96], while NLRP3 activation can also occur in a gingipain-independent manner [103]. Moreover, gingipains enhance the interactions of *P. gingivalis* with other periodontal pathogens [54].

LtxA of *A. actinomycetemcomitans* affects leukocyte populations by activating neutrophil degranulation, causing a massive release of lysosomal enzymes, net-like structures, and matrix metalloproteinases (MMP) and by the induction of apoptosis in lymphocytes [104]. The inhibition of caspase-1 prevents LtxA-mediated cell death in monocytes, suggesting a critical role of pyroptosis to its excecution [105]. *A. actinomycetemcomitans* may also enhance NLRP3 inflammasome expression, irrespective of its major virulence factors [106].

There is a mutual interaction of different virulence factors from different types of bacteria in a coordinated manner [98,104]. Thus, care should be taken when interpreting the observations made of a single virulence factor. Indeed, the complexity of the subgingival biofilm to modulate NLRP3 and IL-1β in cells would require a simulated biofilm in vitro [107]. For instance, based on biofilm research, we can learn that *P. gingivalis* activates the inflammasome to produce IL-1β, whereas others state that *P. gingivalis* inhibits the inflammasome [108]. Future research should thus consider the complexity of the native biofilm with its large spectrum of virulence factors originating from *P. gingivalis*, *A. actinomycetemcomitans*, *T. denticola*, *F. nucleatum*, *T. forsythia*, and other microbial pathogens of the oral biofilm, with respect to the initiation and propagation of pyroptosis.

Responding rapidly to microbial PAMPs and DAMPs is critical to our innate immune system [31,34,68]. Nevertheless, some non-bacterial related issues may act on the activation of pyroptosis [109]. Clinically, aseptic loosening of artificial joint prostheses is the principal reason that limits the long-term use of this type of rehabilitation. Corrosion products activate macrophages to produce pro-inflammatory cytokines, resulting in local osteolysis [5,86,109], while the wear-induced osteolysis is functionally linked to the NALP3 inflammasome [110,111]. Therefore, it is not exclusively the bacterial virulence factors that activate the inflammasome.

Special attention should be taken since periodontitis was found to exacerbate several systemic diseases, including diabetes [112], cardiovascular disease [113], cancer [114], Alzheimer's disease [115], and other degenerative diseases [116], suggesting a mechanism that involves the dissemination of periodontal pathogens, producing pyroptosis-initiating virulence factors outside the periodontium.

4.2.4. Systemic Disorders Associated with the Periodontal Disease through Pyroptosis

Increasing evidence suggests an association of periodontitis and its keystone pathogen, *P. gingivalis*, with various diseases. For instance, *P. gingivalis* was found to be related with atherosclerosis due to the pyroptosis-related release of IL-1β [17]. In cardiovascular disease, disseminated periopathogens potentially causes the progression of atheroma lesions [83]. For example, caspase-11-gasdermin D-mediated pyroptosis and the subsequent pro-inflammatory response in macrophages are involved in the pathogenesis of atherosclerosis [117], and the selective NLRP3 inhibitor MCC950 hinders atherosclerosis development [118]. When focusing on rheumatoid arthritis, the disseminated *P. gingivalis* and *A. actinomycetemcomitans* may enhance pyroptosis of synovial cells [84]. Early periodontitis may also worsen clinical symptoms in patients with Crohn's disease [85] as *Porphyromonas* strains were identified in the colonic mucosa of patients with ulcerative colitis and Crohn's disease [119]. Taken together, there is reason to assume that periopathogens do not exclusively provoke pyroptosis in periodontal tissues. Periopathogens can disseminate into ectopic sites where they potentially exert their deteriorative activity through pyroptosis activation. Thus, targeting pyroptosis in periodontitis is likely to impact systemic health.

Furthermore, the inflammasomes were also found to be the link among endometriosis, atherosclerosis, periodic fever syndromes, vitiligo, Crohn's disease, gout, asbestosis, silicosis, and periodontitis [70]. In addition, neuroinflammation with pyroptosis is recognised as a pathological factor in Alzheimer's disease [81,82]. Such associations give clues regarding the pathogenic mechanisms involving inflammasomes that are crucial for developing therapies or even for preventing such diseases [70]. Pyroptosis, thus, becomes a target to prevent systemic inflammatory disorders.

4.2.5. Therapeutic Approaches for Pyroptosis-Related Periodontal Disease

The application of pyroptosis inhibitors has been the focus of recent research. MCC950, an NLRP3 specific inhibitor, restored the expression of osteogenic differentiation markers in cells exposed to *E. coli* LPS [10]. Similarly, VX765, a caspase-1 inhibitor, reduced the expressions of IL-1β, in PDLCs stimulated with *E. coli* LPS or *P. gingivalis* LPS, and decreased the inflammatory responses during periodontitis in vivo [3]. Moreover, Z-LEVD-FMK, a caspase-4 specific inhibitor, led to inhibition of GSDMD cleavage, caspase-4 activation, and IL-1β release in a periodontitis rat model [2]. The inhibition of pyroptosis can be indirect as well. For example, by inhibiting cyclin-dependent kinase 9, flavopiridol dampened pyroptosis in the liver and decreased cell death in LPS-exposed monocytes [120]. Additionally, eldecalcitol, a vitamin D analogue, reduced LPS-induced NLRP3 inflammasome-dependent pyroptosis in HGFs via the Nrf2/HO-1 pathway [121]. Thus, direct and indirect pyroptosis inhibitors could help combating periodontal disease [2]. Pharmacological blocking of pyroptosis, however, should be seen with caution as its inhibition must be balanced against its benefits to strengthen the immune system. Clinically, it seems more realistic to remove pathogens and their virulence factors from the periodontal pockets and thereby reduce, or even prevent, pyroptosis-mediated inflammation and tissue damage. In support of the previous affirmation, professional use of local antimicrobial agents, in conjunction with scaling and root debridement, provides significant benefits in periodontal therapy [122]. Moreover, reducing the microbial charge lowers the chance of disseminating periodontal pathogens and their virulence factors into the periphery [114]. Based on this concept, avoiding the dissemination of oral pathogens supports systemic health, avoiding pyroptosis-mediated inflammation and tissue damage.

5. Conclusions

The clinical exacerbated inflammatory processes in periodontitis are yet to be fully understood. The collected data highlights pathways for inflammatory responses that could lead to exacerbated tissue damage and non-responsive therapies in periodontal disease. Pyroptosis is likely to be one of those pathways. Overall, the studies agree that some virulence factors trigger the inflammasome route of caspase-1 activation, which is able to cleave gasdermin D and is also responsible for the maturation and release of interleukins, specifically IL-1β and IL-18. Therefore, pyroptosis is a potential target for periodontal therapy. However, since pyroptosis mainly occurs as a consequence of virulence factors produced by oral pathogens, maintaining oral hygiene is presumably the best strategy to prevent periodontal tissues from pyroptosis-mediated tissue destruction. Finally, it is also important to keep in mind the potential beneficial effects of reducing other inflammatory diseases that are linked with the dissemination of oral pathogens.

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2.5 ARTICLE 5 – ENAMEL MATRIX DERIVATIVE DECREASES PYROPTOSIS-RELATED GENES IN MACROPHAGES

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Article

Enamel Matrix Derivative Decreases Pyroptosis-related Genes in Macrophages

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Abstract

Background: Pyroptosis is a caspase-dependent catabolic process relevant to periodontal disorders for which inflammation is central to the pathophysiology of the disease. Despite enamel matrix derivative (EMD) has been applied to support periodontal regeneration, its capacity to modulate the expression of pyroptosis-related genes remains unknown. Considering EMD has anti-inflammatory properties and pyroptosis is linked to the activation of the inflammasome in chronic periodontitis, the question arises whether EMD could reduce pyroptosis signalling. **Methods:** To answer this question, primary macrophages obtained from murine bone marrow and RAW 264.7 macrophages were primed with EMD before being challenged by lipopolysaccharide (LPS). Cells were then analysed for pyroptosis signalling components by gene expression analyses, interleukin-1β (IL-1β) immunoassay, and the detection of caspase-1 (CAS1). The release of mitochondrial reactive oxygen species (ROS) was also detected. **Results:** We report here that EMD, like the inflammasome (NLRP3) and CAS1 specific inhibitors – MCC950 and Ac-YVAD-cmk, respectively – lowered the LPS-induced expression of NLRP3 in primary macrophages (EMD: p=0.0232; MCC950: p=0.0426; Ac-YVAD-cmk: p=0.0317). EMD further reduced the LPS-induced expression of NLRP3 in RAW 264.7 cells (p=0.0043). There was also a reduction in CAS1 and IL-1β in RAW 264.7 macrophages on the transcriptional level (p=0.0598; p=0.0283; respectively), in IL-1β protein release (p=0.0041), and CAS1 activity. Consistently, EMD, like MCC950 and Ac-YVAD-cmk, diminished ROS release in activated RAW 264.7 cells. In ST2 murine mesenchymal cells, EMD could not be tested because LPS, saliva, and IL-1β+TNF-α failed to provoke pyroptosis signalling. **Conclusion:** These findings suggest that EMD is capable of dampening the expression of pyroptosis-related genes in macrophages.

Keywords: Enamel matrix proteins; pyroptosis; inflammasomes; periodontal diseases; macrophages; mesenchymal cells

1 Introduction

Periodontal disease is a global health problem.[1] Currently, peri-implant disease has reached the same level of concern as periodontal disorders. In this scenario, mucointegration – the attachment of soft tissues to the transmucosal portion of an implant – is just as relevant for implant success as osseointegration.[2] A disruption in mucointegration can manifest as peri-implant mucositis and, if not resolved, can progress to inflammatory peri-implantitis.[3,4] Periodontitis and peri-implantitis are universally agreed to begin with a breakdown in the soft tissue attachment and bone loss progression.[5,6] Consequently, methods to strengthen, maintain, or regenerate the soft tissue attachment around the tooth or the dental implant are critical for improving the protection sealing against microbial infections or endogenous danger signals.[7] The underlying pathogenesis of periodontitis/peri-implantitis is a chronic inflammation that drives downstream catabolic cellular events ultimately leading to tooth loss due to a lack of supporting tissues.[6,8,9] There is thus a critical requirement to understand the fundamental pathological mechanisms on a cellular and molecular basis to implement therapies aiming to regulate inflammation and thereby pave the way for regenerative strategies.[8,9] Thus, understanding the pathways connecting inflammation and tissue destruction will help to develop strategies to prevent and treat periodontitis and peri-implantitis.

Pyroptosis is an inflammatory caspase-dependent catabolic process that is relevant for innate immunity. This process is mainly mediated by the activation of caspase-1 (CAS1) by the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing protein 3 (NLRP3) inflammasome.[10] Then, CAS1 cleaves the gasdermin D (GSDMD), which is responsible for cell membrane perforation and the release of interleukins-1β (IL-1β) and -18 (IL-18),[10] which in turn trigger a robust inflammatory response on the surrounding tissues.[11] NLRP3 and CAS1 are important for bacterial clearance; however, if overexpressed, they may lead to cellular self-destruction, inflammation, and tissue damage.[12] Immunostaining images showed a stronger signalling intensity for NLRP3, cleaved CAS1, and IL-1β in the connective tissue of periodontitis compared to a healthy gingiva.[13] Additionally, using a periodontitis mouse model, higher amounts of NLRP3 and IL-1β were visible in the inflamed gingiva.[13] There is thus evidence for pyroptosis to occur in periodontal diseased tissues.

In vitro periodontal models in pyroptosis research focus on the NLRP3/CAS1/GSDMD-mediated pyroptosis pathway in monocytes, macrophages, and periodontal ligament cells.[11,14–16] NLRP3 inflammasome can react to a wide range of bacterial ligands and play a pivotal role in the pathogenesis of inflammatory diseases. Lipopolysaccharide (LPS) is a virulence factor and a strong agonist of toll-like receptors (TLR) signalling that is able to initiate the downstream of pyroptosis.[16,17] LPS is produced by Gram-negative bacteria.[18] Considering that oral diseases are mainly mediated by Gram-negative bacteria, it makes sense that LPS is related to periodontal disorders.[12,18,19] Taking advantage of this in vitro model, glycogen synthase kinase-3β (GSK-3β) deficiency was identified to lower the LPS-induced pyroptosis through the inactivation of NLRP3 inflammasome.[16] Thus, NLRP3/CAS1/GSDMD-mediated pyroptosis bioassays are suitable to identify components that lower pyroptosis

signalling. Furthermore, considering the impairment caused by pyroptosis on periodontal disorders, finding ways to inhibit or reduce pyroptosis downstream brings prospects for periodontal therapies.

Enamel matrix derivative (EMD) is a xenograft applied to support periodontal regeneration[20] that was also considered a treatment for venous leg ulcers.[21] EMD is an extract of enamel matrix from the tooth germ of piglets and propylene glycol alginate serves as a matrix. Proteome analyses confirmed the presence of enamel matrix proteins amelogenin and ameloblastin,[22] and growth factors such as TGF-β have been also identified.[23,24] More importantly for this paper, EMD has been shown to exert anti-inflammatory activity in vitro. LPS-stimulated rat monocytes exposed to EMD exhibited a decrease in TNF-α production.[25] In human blood-derived cells exposed to LPS and peptidoglycan, EMD lowered TNF-α release.[26] Furthermore, in LPS-stimulated human osteogenic cells and immortalized human epithelial gingival keratinocytes, EMD lowered the expression of inflammatory cytokines including TNF-α.[27] Nevertheless, the expression of pyroptosis factors in cells stimulated with pyroptosis-triggering dangers – and primed with EMD – was not yet explored. It might be hypothesized that the beneficial effects of EMD[25–27] are caused by lowering the pyroptosis-mediated cellular self-destruction and inflammation in periodontitis.

Since there is strong in vitro evidence that EMD has anti-inflammatory properties[25–27] and pyroptosis is linked to the activation of the inflammasome in chronic periodontitis and peri-implantitis,[11,12,16] the question arises whether EMD could reduce pyroptosis in vitro. Therefore, we tested the hypothesis that the anti-inflammatory activity of EMD is at least partially involving a lowering of the LPS-mediated pyroptosis factors.

2. Materials and methods

2.1. Primary macrophages, RAW 264.7 macrophage-like cells, and ST2 mesenchymal cells

BALB/c mice of 6- to 8-weeks old were purchased from Animal Research Laboratories, Himberg, Austria. Bone marrow cells were collected from the femora and tibiae as previously described.[28] Briefly, mice were sacrificed, and the femora and tibiae were removed. Bone marrow cells were seeded at 1×10^6 cells/cm^2 into 24-well plates and grown for 7 days in Dulbecco's Modified Essential Medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Capricorn Scientific GmbH, Ebsdorfergrund, Germany), 1% antibiotics (PS; Sigma Aldrich, St. Louis, MO, USA), and 20 ng/mL macrophage colony-stimulating factor (M-CSF; ProSpec, Ness-Ziona, Israel). RAW 264.7 macrophage-like cells (LGC Standards, Wesel, Germany) were expanded in growth medium and seeded at 3×10^5 cells/cm^2 into 24-well plates. ST2 murine mesenchymal cells (RIKEN Cell Bank, Tsukuba, Japan) isolated from mouse bone marrow were seeded at 3×10^5 cells/cm^2 into 24-well plates. Cells were primed with 30 μg/mL of enamel derivative matrix (EMD; Straumann AG, Switzerland) for 1 h and then exposed to 100 ng/mL of LPS from *Escherichia coli* 055:B5 (Sigma Aldrich, St. Louis, MO, USA) for 6 h to induce an inflammatory response.

Alternatively, 5% saliva[29] or 20 ng/mL IL-1β (ProSpec, Ness-Ziona, Israel) and TNF-α (ProSpec, Ness-Ziona, Israel) were used for cell stimulation. Pyroptosis-specific inhibitors were applied to establish the in vitro LPS-induced pyroptosis model. MCC950 (CP-456773 Sodium, Selleck Chemicals GmbH, Houston, USA) was applied at 8 μM for 30 min before cells were exposed to LPS. Ac-YVAD-cmk (≥95%, HPLC; Sigma Aldrich, St. Louis, MO, USA) was applied at 5 μM for 20 h prior LPS challenge. All cell lineages were exposed to the respective treatments under standard conditions at 37°C, 5% CO_2, and 95% humidity.

2.2. Reverse Transcription Quantitative Real-Time PCR (RT-qPCR) and immunoassay

For RT-qPCR, after stimulation, total RNA was isolated with the ExtractMe total RNA kit (Blirt S.A., Gdańsk, Poland) followed by reverse transcription and polymerase chain reaction (LabQ, Labconsulting, Vienna, Austria) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA levels were calculated by normalizing to the housekeeping gene GAPDH using the $^{\Delta\Delta}$Ct method.

The primer sequences were:
mNLRP3-F: TCACAACTCGCCCAAGGAGGAA;
mNLRP3-R: AAGAGACCACGGCAGAAGCTAG;
mCAS1-F: GGCACATTTCCAGGACTGACTG;
mCAS1-R: GCAAGACGTGTACGAGTGGTTG;
mCAS11-F: CCTGAAGAGTTCACAAGGCTT;
mCAS11-R: CCTTTCGTGTAGGGCCATTG;
mGSDMD-F: GGTGCTTGACTCTGGAGAACTG;
mGSDMD-R: GCTGCTTTGACAGCACCGTTGT;
mIL-1β-F: CAACCAACAAGTGATATTCTCCATG;
mIL-1β-R: GATCCACACTCTCCAGCTGCA;
mIL-18-F: CAAACCTTCCAAATCACTTCCT;
mIL-18-R: TCCTTGAAGTTGACGCAAGA;
mGAPDH-F: AACTTTGGCATTGTGGAAGG;
mGAPDH-R: GGATGCAGGGATGATGTTCT.

RT-PCR data are represented compared to the untreated control. Supernatants and the respective cell lysates prepared with 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) were analysed for IL-1β secretion by immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

2.3. Western blot

RAW 264.7 cells were seeded at 1 × 10^6 cells/cm^3 into 12-well plates. The following day serum-starved cells were primed with EMD for 1 h and then exposed to LPS for another 6 h. Extracts containing SDS buffer with protease and phosphatase inhibitors were separated by SDS-PAG (cOmplete ULTRA Tablets and PhosSTOP; Roche, Mannheim, Germany) and transferred onto PVDF membranes (Roche Diagnostics, Mannheim, Germany). Membranes were blocked and the binding of the Caspase-1 (D7F10), Gasdermin D (E8G3F), and cleaved Gasdermin D (E7H9G) first antibodies (rabbit IgG, 1:1000; Cell Signaling Technology, Danvers, MA, USA) were detected with the second antibody labelled with HRP (goat anti-rabbit IgG, 1:10000; Cell Signaling Technology, Danvers, MA, USA).

After exposure to the Clarity Western ECL Substrate (Bio-Rad Laboratories Inc., Hercules, CA, USA) chemiluminescence signals were visualized with the ChemiDoc imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Quantification of bands intensity was performed using ImageJ software.

2.4. Mitochondrial reactive oxygen species (ROS) release

RAW 264.7 cells were seeded at 3×10^5 cells/cm^2 into 96-well plates and followed the standard stimulation with EMD, MCC950, or Ac-YVAD-cmk, then challenged with LPS for 6 h. Cells were analysed for the release of mitochondrial reactive oxygen species (MitoROS™ 580, AAT Bioquest, Inc., Sunnyvale, USA) according to the manufacturer's instructions.

2.5. Statistical analysis

All experiments were performed at least three times. Statistical analyses of gene expression and immunoassays were performed with paired t-test, while ROS release statistical analyses were performed with one-way ANOVA followed by Dunnett's multiple comparison test. Analyses were performed using Prism v.9 (GraphPad Software, La Jolla, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Pyroptosis inhibitors validate macrophages to serve as a pyroptosis model

To establish a pyroptosis model, primary macrophages generated from murine bone marrow were exposed to *E. coli* LPS. MCC950 and Ac-YVAD-cmk were introduced as inhibitors raised against NLRP3 and CAS1, respectively. MCC950 reduced the forced expression of NLRP3, CAS11, and IL-1β, but also a reduction in CAS1 and IL-18, in primary macrophages. Likewise, Ac-YVAD-cmk reduced the forced expression of NLRP3 and IL-18, and showed a trend to the reduction in the expression of CAS1, CAS11, and IL-1β, in primary macrophages (Figure 1). These findings support the LPS-induced primary macrophages to serve as a bioassay to test EMD and its potential for reducing pyroptosis signalling.

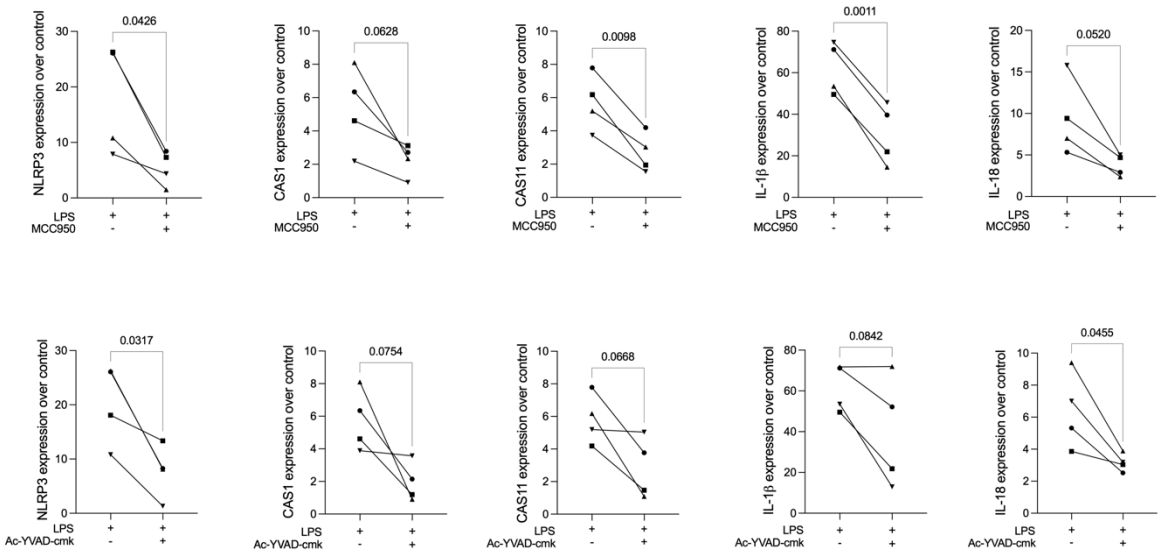

Figure 1. LPS stimulation caused an increase in the expression of the pyroptosis genes NLRP3, CAS1, CAS11, IL-1β, and IL-18 in primary macrophages. The application of MCC950 prior to LPS stimulation in primary macrophages led to a significant reduction in the expression of NLRP3, CAS11, and IL-1β, and a trend in the reduction of CAS1 and IL-18. The application of Ac-YVAD-cmk prior to LPS stimulation in primary macrophages led to a reduction in the forced expression of NLRP3 and IL-18, and a trend in the reduction of CAS1, CAS11, and IL-1β. Different symbol shapes mean independent experiments. Paired t-test statistical analysis was applied to compare the groups.

3.2. EMD reduces the expression of pyroptosis markers in LPS-induced primary macrophages

To test EMD and its potential for reducing pyroptosis in the established bioassay, primary macrophages were primed with EMD before being challenged by LPS and then analysed for gene expression of pyroptosis signalling components. Our chosen dose of 30 μg/mL EMD did not lead to any cytotoxicity either alone or in combination with LPS (data not shown); therefore, proceed with the gene expression analyses. LPS caused a robust increase in the expression of the pyroptosis genes NLRP3, CAS1, CAS11, IL-1β, and IL-18 in primary macrophages, with a particularly strong increase in NLRP3 and IL-1β. EMD significantly lowered the LPS-induced expression of NLRP3, CAS1, and IL-18, suggesting that primary macrophages are susceptible to EMD and its pyroptosis-lowering activity (Figure 2).

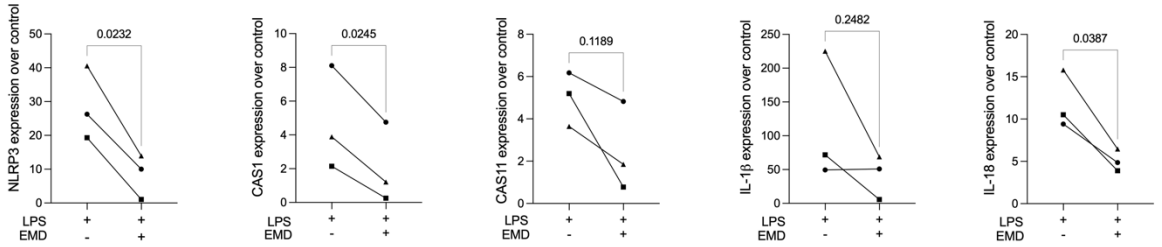

Figure 2. LPS stimulation caused an increase in the expression of the pyroptosis genes in primary macrophages. The application of EMD prior to LPS stimulation in primary macrophages led to a reduction in the forced expression of NLRP3, CAS1, and IL-18, and showed a trend in the reduction of CAS11 and IL-1β, in primary macrophages. Different symbol shapes mean independent experiments. Paired t-test statistical analysis was applied to compare the groups.

3.3. EMD reduces the expression of pyroptosis markers in LPS-induced RAW 264.7 macrophages

To implement a cell line-based pyroptosis model, RAW 264.7 macrophages were exposed to LPS followed by the screening for the respective pyroptosis marker genes. Consistent with the findings regarding the primary macrophages, EMD significantly reduced the LPS-induced expression of NLRP3, CAS1, and IL-1β. There was also a trend toward reducing CAS11 expression (Figure 3). Differently from primary macrophages though, it was mainly the IL-1β but not the IL-18 expression that was reduced by EMD in RAW 264.7 cells. As expected,[30] immunoassays revealed negligible amounts of IL-1β in the supernatant (Supplement Figure 1A). Nevertheless, under the permeabilization of the cell membrane, IL-1β could be confirmed in LPS-stimulated RAW 264.7 cells as well as the significant IL-1β reduction with the treatment with EMD (Figure 4A). Moreover, EMD reduced cleaved CAS1 at the protein level (Figure 4B), suggesting a decrease in the CAS1 activity and that EMD could lower the expression and the activation of CAS1 by NLRP3 reduction. The bands were quantified regarding intensity (Supplement Figure 2), confirming what can be pictured in the Western blot images. Thus, the RAW 264.7 macrophages are suitable to identify EMD for lowering a pyroptosis response.

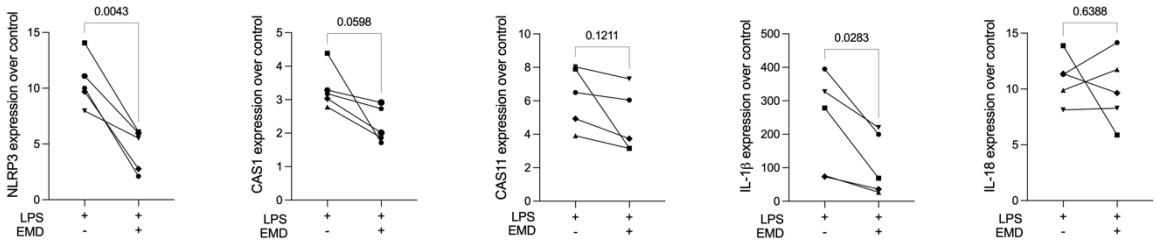

Figure 3. LPS stimulation caused an increase in the expression of the pyroptosis genes in RAW 264.7 macrophages. The application of EMD prior to LPS stimulation in RAW 264.7 cells led to a significant reduction in the forced expression of NLRP3 and IL-1β, and a trend in the reduction of CAS1 and CAS11. Different symbol shapes mean independent experiments. Paired t-test statistical analysis was applied to compare the groups.

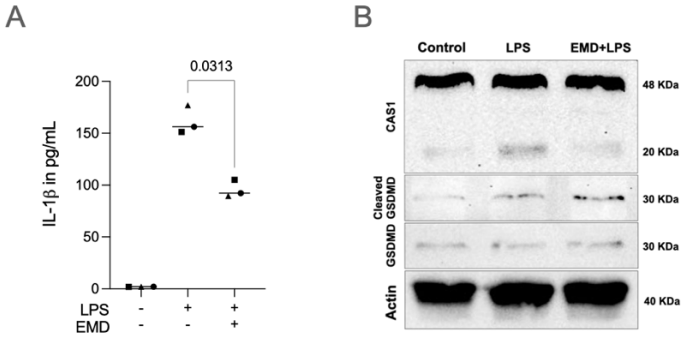

Figure 4. EMD reduces the pyroptosis factors in LPS-induced RAW 264.7 macrophages. (A) EMD protection on LPS-stimulated RAW 264.7 macrophages led to IL-1β reduction detected from the immunoassay. Different symbol shapes mean independent experiments. Paired t-test to compare LPS and EMD+LPS groups was applied. (B) Confirming the gene expression, Western blot analysis showed

less cleaved CAS1 (20 KDa) protein expression in RAW 264.7 cells primed with EMD. Cleaved GSDMD was present for cells stimulated with LPS and GSDMD was present for all cells.

3.4. EMD reduces reactive oxygen species (ROS) in LPS-induced RAW 264.7 macrophages

RAW 264.7 macrophages were again exposed to LPS and analysed for mitochondrial ROS release. EMD reduced the mitochondrial ROS release in RAW 264.7 cells to levels comparable to the untreated control, suggesting a reduction in cellular stress levels by the EMD treatment. Consistently, the pyroptosis specific inhibitors, MCC950 and Ac-YVAD-cmk, diminished ROS release in activated RAW 264.7 cells (Figure 5).

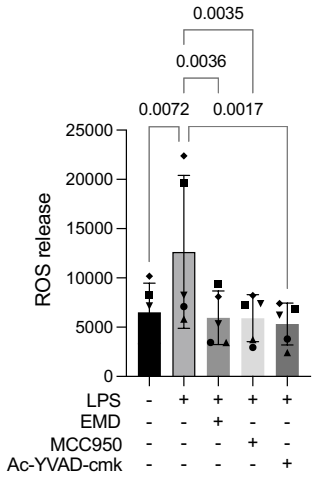

Figure 5. LPS stimulation caused an increase in the reactive oxygen species (ROS) release in RAW 364.7 macrophages. The application of EMD, MCC950, or Ac-YVAD-cmk in LPS-induced RAW 264.7 cells showed a trend in the reduction of ROS release. Different symbol shapes mean independent experiments. Repeated measures one-way ANOVA followed by Dunnett's multiple comparison test, comparing every group to the LPS group, was applied.

3.5. ST2 mesenchymal cells are not suitable to test for a potential role of EMD on pyroptosis

Finally, we introduced LPS and saliva stimulation over ST2 murine mesenchymal cells to serve as a model for pyroptosis testing. However, LPS or saliva failed to considerably increase the expression of the most sensitive pyroptosis marker – NLRP3 – and all other pyroptosis markers, including the IL-1β and IL-18, suggesting that LPS or saliva stimulation in ST2 cells are not suitable as a model to evaluate EMD to change pyroptosis (Supplement Figure 2). When ST2 cells were exposed to IL-1β and TNF-α, there was a strong increase of interleukin-6 and chemokines CCL2 and CXCL2 that was reduced by EMD (Figure 6). Nevertheless, no changes in NLRP3 and all other pyroptosis markers were found (Supplement Figure 3). Thus, LPS, saliva, or IL-1β+TNF-α challenging to ST2 cells are not applicable to evaluate the potential role of EMD to reduce pyroptosis signalling.

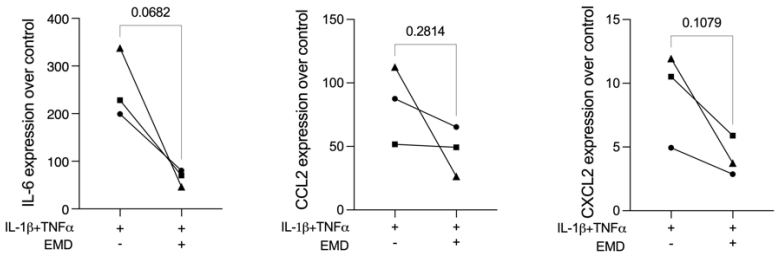

Figure 6. IL-1β and TNF-α stimulation caused a strong expression of the interleukin IL-6 and chemokines CCL2 and CXCL2 in ST2 cells. The application of EMD prior to IL-1β and TNF-α stimulation in ST2 cells led to a trend in the reduction of the forced expression of inflammatory markers (IL-6, CCL2, and CXCL2). Different symbol shapes mean independent experiments. Paired t-test statistical analysis was applied to compare the groups.

4. Discussion

Pyroptosis is a major driver of inflammatory disorders, and it is chiefly activated by NLRP3 inflammasome and caspases. Thus, NLRP3 and CAS1, the hallmarks of pyroptosis signalling, are increasingly expressed in periodontal disease compared to healthy tissue.[10,12,31] Considering EMD is widely used in periodontal regeneration and has demonstrated anti-inflammatory properties in vitro,[20,25–27] we hypothesized that part of the beneficial activity of EMD might involve the modulation of pyroptosis signalling. Indeed, our major finding was that EMD lowered the forced expression of NLRP3 and CAS1 activity in murine macrophage models. Taken together, our findings suggest that EMD diminishes pyroptosis signalling in macrophages.

If we relate our findings to those of other studies, our data completes the overall picture of the anti-inflammatory activity that EMD has in vitro on various models from human, rat, and mouse cells.[25–27,32] However, these models mainly used TNFα or interferon-gamma (IFNγ) to simulate inflammation, but TNFα and IFNγ are not drivers of pyroptosis signalling. It was only the study on LPS-stimulated human osteoblastic cells and human gingival keratinocytes that studied EMD lowering the expression of IL-1β and it was not focused on pyroptosis.[27] Hence, our findings that EMD reduces the expression of IL-1β in RAW 264.7 macrophages support the existing knowledge on the anti-inflammatory properties of EMD while this observation is not sufficient to support the involvement of EMD in the reduction of the pyroptosis signalling.

Macrophages can be polarized into either classically activated pro-inflammatory (M1) or alternatively activated (M2) anti-inflammatory macrophages depending on the stimulation.[33] The M1 macrophages are induced by pathogen-associated molecular patterns (PAMPs), such as the bacterial LPS used herein, or Th1 cytokines such as IFNγ, producing a wide range of cytokines, such as TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS), to aggravate inflammation. In contrast, the M2 macrophages are induced by Th2 cytokines such as IL-4 and IL-13, and they possess the ability to express arginase-1 (Arg1), chitinase-like 3 (or Ym1), and IL-10 to promote reparative processes and relieve inflammation.[34] Therefore, since we have applied LPS, we know we are

working with M1 pro-inflammatory macrophages. Furthermore, since this is a pyroptosis related article, we did not focus on IFNγ, TNF-α, IL-6, or iNOS, but on IL-1β and IL-18, which are directly related to pyroptosis. Moreover, the production of ROS is a hallmark of M1 macrophages, which also contributes to the M2 polarization switch.[35] Nevertheless, since we have scientific support that we are working with M1 macrophages, the release of ROS analyses is related to the pro-inflammatory aspect.

Our data showing that EMD significantly reduced the expression of IL-18 in primary macrophages and that NLRP3 and CAS1 specific inhibitors (MCC950 and Ac-YVAD-cmk, respectively) exert a similar activity, can be considered indirect support for EMD to attenuate pyroptosis activity. These findings are in line with other observations showing that MCC950 inhibited IL-18 release in THP1 and monocytes,[36,37] reversed the forced IL-1β and IL-18 expression on periodontal ligament fibroblasts,[38] HCT116 colorectal cells,[39] and canine kidney epithelial cells.[40] Also, Ac-YVAD-cmk reduced the forced expression of IL-18 in whole blood cells,[41] in THP-1 cells,[42] and also in sepsis-induced acute kidney injury.[43] Even though EMD performs similarly to MCC950 and Ac-YVAD-cmk inhibitors, and reduces the expression of NLRP3, CAS1, and IL-18 in primary macrophages, this is not sufficient evidence that EMD reduces pyroptosis activity and should be supported by additional investigation.

Support for EMD to regulate pyroptosis arises from findings that EMD reduces the LPS-induced expression of NLRP3 and IL-1β in RAW 264.7 macrophages. Considering that NLRP3 together with IL-1β and IL-18 are NF-kB-target genes, it can be hypothesized that EMD lowers the LPS-driven NF-kB signalling pathway and thereby the transcription of NLRP3 and IL-1β/IL-18. Consequently, the assembly of the inflammasome is limited by the accessibility of the reduced NLRP3, and our observation that EMD lowers the LPS-induced CAS1 activity supports this concept. Thus, our findings add to the existing knowledge on the anti-inflammatory properties of EMD and encompass them towards the regulation of the pyroptosis pathway in macrophages. Furthermore, our data on EMD reducing inflammation in ST2 challenged cells also supports the anti-inflammatory potential of EMD in vitro.

Consistent with other reports,[30] immunoassays failed in detecting IL-1β in the extracellular media in LPS-stimulated RAW 264.7 cells while, after cell membrane permeabilization, IL-1β was able to be detected. This seems to be related to the weak GSDMD activity that is herein reported. GSDMD is required for IL-1β release in pyroptotic cells or hyperactivated cells.[30] GSDMD knockout cells were unable to form pores and release IL-1β or lactate dehydrogenase (LDH), a molecule that shows signs of membrane pore formation.[30] This agrees with our finding that LDH release was not substantially increased in LPS-stimulated RAW 264.7 macrophages (Supplement Figure 1B). Furthermore, GSDMD is necessary for the release of cleaved IL-1β during infection but is not required for IL-1β processing within cells.[30] Hence, it seems like our model failed to cause membrane pore formation due to reduced GSDMD activity. Thus, our model is valid to test for pyroptosis signalling but not for full pyroptosis induction including membrane disintegration.

Regarding ROS release, EMD in LPS-stimulated RAW 264.7 macrophages reduced mitochondrial ROS, like the NLRP3 and CAS1 specific inhibitors (MCC950 and Ac-YVAD-cmk, respectively). In agreement with our findings, MCC950 inhibited the excessive production

of ROS in chondrocytes,[44] and Ac-YVAD-cmk blocked the forced ROS production in HT22 cells[45] and cerebellar granule neurons[46]. Increased ROS levels drive the transcription nuclear factor and induce the pyroptosis of nucleus pulposus cells through the NLRP3 pathway, which is related to the mechanism of degenerative disorders.[47] More importantly, ROS acts downstream of gene transcription, mRNA translation, and IL-1β converting enzyme activation.[46] Also, ROS production occurs after K^+ deprivation,[30,46] which can induce pyroptosis.[12] Therefore, evaluating ROS release is relevant to pyroptosis signalling as part of the downstream events occurring in the pyroptotic cells.

The clinical relevance of our findings remains at the level of speculation. Clinically, EMD stabilizes blood clots and improves clinical healing in deep pockets after non-surgical periodontal treatment.[48] Minimally invasive periodontal surgery with EMD in periodontitis-affected subjects results in lower values of C-reactive protein as no inflammatory perturbation was noticed.[49] Also, EMD treatment reduced bleeding on probing and periodontal pockets depth, and post-surgical gingival recession was lowered.[49] Moreover, EMD shows an antibacterial effect on the viability of ex vivo supragingival dental plaque flora collected from patients with periodontitis.[50] Considering that EMD lowers the inflammation also in vivo[51] and that periodontitis is linked to pyroptosis,[11,12,16] we can speculate that EMD exerts its beneficial effect by reducing pyroptosis signalling at sites of chronic periodontitis, likely involving the NLRP3 expression.

The complexity of the in vivo situation, however, is not fully represented by primary macrophages or cell lines. Primary macrophages are closer to the in vivo situation than cell lines and, therefore, we have used the primary macrophages to establish the pyroptosis system and to perform the proof-of-principle experiments. However, to reduce and replace animal organ donation – in this case, bone marrow – once we had established our model with the use of pyroptosis-specific inhibitors and evidence that EMD reduces pyroptosis-related genes, we switched to a macrophage cell line. As expected, the cell line performed similar although not identical to the primary cell line. Furthermore, in vitro models are useful to identify potential cellular responses and signalling pathways that can later be evaluated in a complex in vivo environment. By showing that EMD lowers the LPS-induced expression of pyroptosis-related genes, we provide a fundament for future research in this direction.

This study has the limitations of the in vitro research. For instance, what and how EMD component molecules responsible for the anti-pyroptosis activity reach the target cells in vivo was not explored. Once we have not discovered the molecular structure and the characteristics of the anti-inflammatory components of EMD, the in vitro findings cannot easily be translated to a clinical perspective. Hence, further studies of EMD in the inhibition of pyroptosis in periodontal tissues should be conducted in vivo. Since EMD is available for clinical purposes, studies on its impact on the periodontium are feasible. Another limitation of our model is that LPS alone is not sufficient to increase the expression or activate GSDMD, an executor of pyroptosis and required for the IL-1β secretion in macrophages.[52] Future studies could therefore include pyroptosis agonists such as α-hemolysin,[53,54] nigericin,[30] or ATP,[55] together

with LPS to impulse cytotoxicity and IL-1β secretion other than the gene expression of pyroptosis-related factors, i.e. the full picture of pyroptosis.[52] It might also be worth considering the impact of EMD on the CAS3 dependent apoptotic pathway, downstream of CAS1 and independent of GSDMD.[56] Further proof for EMD to reduce pyroptosis-mediated periodontal destructing might be based on mouse models with genetic deletion of CAS1 and GSDMD; hypothetically, EMD cannot exert its beneficial activity when pyroptosis is blocked at the genetic level.

In conclusion, our findings suggest that EMD is capable of dampening pyroptosis-related genes in macrophages. This is relevant as the clinical use of EMD in periodontal therapies might comprise the reduction of pyroptosis downstream under conditions of periodontal tissue inflammation.

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Ethical statement for care and use of animals: The bone marrow cells were collected from the femora and tibiae of BALB/c mice, which were purchased from Animal Research Laboratories, Himberg, Austria. According to Austrian law, organ donation from mice required an informal approval of the local veterinarian authorities but not a formal approval by the Ethics Committee.

Conflicts of Interest: The authors state no conflicts of interest related to this project.

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3 CONCLUDING REMARKS

The present thesis allowed to draw two main conclusions regarding the main objectives of this project and some insight for future research.

First, scaffolds designed for bone tissue engineering are difficult to be produced but are promising for clinical applications considering the emerging technologies, especially three-dimensional bioprinting, that permit a reproducible manner to fabricate scaffolds together with cells and growth factors. Abundant scientific research has been published in the field of bone tissue augmentation strategies; nevertheless, none has successfully achieved clinical application and long-term maintenance. Apart from the difficulty to regenerate a complex tissue such as bone, this is probably due to the enormous gap between *in vitro* research and *in vivo* conditions, especially regarding cell behaviour. Therefore, at this point, it is imperative to discuss the reasoning that prevents all the efforts made to develop scaffolds for bone tissue engineering to be translated into clinical application. First of all, a better understanding of the cell transitions from a quiescent and undifferentiated state up to the point that they respond to stimuli that allow them to differentiate into the desired lineage is mandatory. This is because the cells are the key for regenerative medicine and tissue engineering therapies. In addition, understanding cell transitions will give insights not only for craniofacial bone tissue regeneration but also for other fields throughout the human organism and additionally give responses to unmet demands on cell biology.

Second, pyroptosis is a pathway of inflammatory diseases that have been recently raised as a concern in dentistry, especially in periodontal disorders. Nevertheless, some anti-inflammatory compounds are promising as potential substances to dampen the exacerbation of pyroptosis in periodontal tissues. Since there is strong *in vitro* evidence that enamel derivative matrix has anti-inflammatory properties, it was proposed and successfully disclosed that such matrix has potential to reduce pyroptosis-related factors in inflammatory macrophages. Nevertheless, this was a very preliminary data finding in murine cell lineages. Further ways to induce pyroptosis downstream are required since the present model failed to fully provoke pyroptosis cascade. Also, the introduction of other human oral cell lineages, such epithelial and fibroblastic cells, are required prior to the translation to *in vivo* research. However, it was the first time that enamel derivative matrix was linked to pyroptosis on an oral biology approach. This gives plenty of insights for further research and open space for the experimentation of

other substances that could be applied to reduce or inhibit pyroptosis exacerbation. Furthermore, understanding pyroptosis in an oral environment approach is fundamental to finding clinical solutions for periodontal and peri-implantar disorders.

Finally, the broad and main conclusion that might be taken from all the pieces of research presented in this thesis project is that science luckily will certainly not stop evolving and scientists should be open to criticising their own research in order to move forward. After long years of post-graduation, the articles presented herein are just pieces of a puzzle resulting from personal and professional development made on a scientific journey.

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