UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GABRIELLE CHRISTINE BONETTI SALLUM

DESENVOLVIMENTO DE *SCAFFOLD* LAMELAR DENSO À BASE DE QUITOSANA, COLÁGENO TIPO 1 E ÁCIDO HIALURÔNICO PARA FORMAÇÃO DE MATRIZ ÓSSEA

DEVELOPMENT OF DENSE LAMELLAR *SCAFFOLD* BASED ON CHITOSAN, COLLAGEN TYPE 1 AND HYALURONIC ACID FOR BONE MATRIX FORMATION

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Mestra em Clínica Odontológica, na Área de Periodontia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of requirements for the degree of Master in Clinical Dentistry, in Periodontology area.

Orientadora: Profª. Drª. Karina Gonzales Silverio Ruiz

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“We keep moving forward, opening new doors, and doing new things, because we’re curious and curiosity keeps leading us down new paths.”

- Walter Elias Disney

RESUMO

Cirurgias regenerativas que utilizam enxertos ósseos e seus substitutos são frequentemente incluídas no plano de tratamento de pacientes totalmente ou parcialmente edêntulos, quando a estrutura óssea não é suficiente em termos de qualidade e volume para a instalação satisfatória de implantes. Por isso, a engenharia de tecidos tem ganhado interesse crescente, com o desenvolvimento de novos *scaffolds* para regeneração óssea. Assim, este estudo in vitro teve como objetivo avaliar a biocompatibilidade e as propriedades indutoras osteogênicas de um *scaffold* composto por quitosana (Qt), colágeno tipo 1 (COL-1) e ácido hialurônico (AH). Dois grupos foram considerados usando células imortalizadas pré-osteoblásticas obtidas da calvária de camundongos (células MC3T3-E1) para cultura celular: 1) Grupo controle, onde as células foram cultivadas diretamente em placas de cultura, e 2) Grupo Qt+COL-1+AH, onde as células foram cultivadas diretamente no *scaffold*. A viabilidade celular foi avaliada usando o ensaio MTS (3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4-sulfofenil)-2H-tetrazólio); a capacidade de adesão e disseminação celular foi examinada por meio de análise de microscopia eletrônica de varredura (MEV); a indução da formação de matriz mineralizada foi avaliada com o ensaio de coloração de vermelho de alizarina (AR-S); a maturação celular em um fenótipo osteoblástico foi medida pela quantificação da atividade da enzima fosfatase alcalina (ALP) e pela expressão gênica de Runx2 (Runt-related transcription factor) e Ocn (osteocalcina). Os resultados mostraram que as células permaneceram viáveis durante todo o ensaio MTS, no entanto, não houve diferenças estatisticamente significativas entre os grupos na viabilidade celular após 1, 3 e 5 dias de cultura, conforme determinado pelo ensaio MTS. A análise em MEV revelou a presença limitada de células no *scaffold* após 48 horas de carreamento celular, sugerindo que as células ainda estavam na fase de adesão, em vez de proliferar ativamente no biomaterial. Notavelmente, o grupo de *scaffold* exibiu um aumento significativo na formação de nódulos minerais em comparação com o grupo controle ($p < 0,05$) após 14 dias, e apresentou níveis mais baixos de ALP em comparação com o grupo controle após 7 e 10 dias. Os níveis de

Runx2 foram significativamente aumentados nos dias 10 (em meio padrão) e 14 (em meio osteogênico) para o *scaffold*. Além disso, os níveis de RNA para o gene Ocn foram maiores no grupo de *scaffolds* nos dias 10 e 14, em ambos os meios de cultura. Dentro das limitações deste estudo, o *scaffold* composto de Qt, COL-1 e AH demonstrou potencial promissor para futuras abordagens para regeneração óssea. No entanto, melhorias adicionais são necessárias para aumentar a adesão celular e disseminação no *scaffold*.

Palavras-chave: biopolímeros; colágeno tipo 1; quitosana; ácido hialurônico; osteoblasto; mineralização.

ABSTRACT

Guided regenerative surgeries using bone grafts and their substitutes are often included in the treatment plan of totally or partially edentulous patients, when the bone structure is not sufficient in terms of quality and volume for the implants to be installed satisfactorily. For that reason, tissue engineering has gained increasing interest, with the development of new *scaffolds* for bone regeneration. Thus, this in vitro study aimed to evaluate the biocompatibility and osteogenic inducing properties of a *scaffold* composed of chitosan (CH), collagen type 1 (COL-1), and hyaluronic acid (HA). Two groups were considered using pre-osteoblastic immortalized cells of a murine bone calvaria (MC3T3-E1 cells) for cell culture: 1) Control group, where cells were cultured directly on polystyrene plates, and 2) *CH+COL-1+AH* group, where cells were cultured in the *scaffold*. Cell viability was evaluated using the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); cell adhesion and spreading capacity was examined through scanning electron microscopy analysis (SEM); the induction of mineralized matrix formation was evaluated with alizarin red staining (AR-S) assay; cell maturation into an osteoblastic phenotype was measured by the quantification of the activity of alkaline phosphatase enzyme (ALP), and by gene expression of Runx2 (Runt-related transcription factor) and Ocn (osteocalcin). Results showed that the cells remained viable throughtout the MTS assay, however, there were no statistically significant differences between the groups in cell viability after 1, 3, and 5 days of culture as determined by the MTS assay. SEM analysis revealed limited cell presence in the *scaffold* after 48 hours of loading, suggesting that the cells were still in the adhesion phase rather than actively proliferating on the biomaterial. Notably, the *scaffold* group exhibited a significant increase in mineral nodule formation compared to the control group ($p < 0.05$) after 14 days, and also displayed lower levels of ALP compared to the control group after 7 and 10 days. The levels of Runx2 were significantly upregulated at days 10 (in standard medium) and 14 (in osteogenic medium) for the *scaffold*. Furthermore, the levels of RNA for the Ocn gene were higher in the *scaffold* group at days 10 and 14, in both culture media. Within the limitations of this study, the *scaffold* composed of CH, COL-1, and HA demonstrated

promising potential for future bone regenerative approaches. However, further improvements are necessary to enhance cell adhesion and spreading on the *scaffold*.

Keywords: biopolymers; collagen type 1; chitosan; hialuronic acid; osteoblast; mineralization.

SUMÁRIO

1 INTRODUÇÃO

No cenário atual, os implantes dentários têm se consolidado como uma terapia previsível e de longo prazo, oferecendo uma opção viável e vantajosa para pacientes totais ou parcialmente edêntulos (ADELL et al., 1990). No entanto, a reabilitação com implantes requer uma estrutura óssea com qualidade, densidade e volume adequados, garantindo a instalação satisfatória dos implantes e subsequente reabilitação protética. Diante dessa necessidade, cirurgias regenerativas com enxertos ósseos e substitutos são incluídas no plano de tratamento, uma vez que, sem a realização de reconstruções ósseas, a reabilitação com implantes fica inviável em muitos casos.

Nesse contexto, os materiais de enxertia têm desempenhado um papel fundamental na odontologia regenerativa há décadas (HOEXTER, 2002). Dentre as possibilidades de enxertos ósseos disponíveis temos os enxertos autógenos, enxertos alógenos, enxertos xenógenos e materiais aloplásticos (HOEXTER, 2002). Os enxertos autógenos são aqueles extraídos de um sítio doador e transplantados para outra região do mesmo indivíduo, e são considerados o padrão-ouro devido à baixa imunogenicidade e às propriedades osteoindutoras e osteocondutoras, resultantes da preservação de células viáveis e de fatores de crescimento (PILIPCHUK et al., 2015). No entanto, estão associados a desvantagens como a necessidade de uma área cirúrgica adicional para a remoção do enxerto, aumento da morbidade e complicações pós-operatórias, bem como a limitação de disponibilidade óssea em defeitos extensos (PILIPCHUK et al., 2015; SEOL et al., 2014). Portanto, um dos maiores desafios da odontologia contemporânea tem sido a busca por biomateriais que possam substituir o osso autógeno de maneira previsível. Há no mercado, uma grande disponibilidade de biomateriais que apresentam vantagens e desvantagens com relação ao uso para substituição do tecido ósseo. Entretanto, ainda se busca um material ideal, que possa ser capaz de devolver anatomia e função semelhantes às do tecido ósseo perdido, apresentando as propriedades semelhantes do enxerto ósseo autógeno.

A engenharia tecidual tem emergido com o objetivo de ampliar a previsibilidade e as aplicações dos procedimentos regenerativos, com o objetivo de criar condições "ideais" para a regeneração tecidual por meio da

interação de três elementos-chave: células, matriz carreadora e moléculas sinalizadoras (Bartold et al., 2000). Dentro dessa perspectiva, a engenharia tecidual voltada para a neoformação óssea se baseia em quatro fatores cruciais: 1) o uso de uma matriz tridimensional biocompatível, mimetizando a matriz óssea extracelular; 2) a adesão e proliferação de células osteogênicas na superfície da matriz; 3) a presença de sinalização morfogênica que estimula a diferenciação celular para o fenótipo osteogênico; e 4) a promoção da neovascularização, fornecendo nutrientes essenciais ao crescimento tecidual e removendo catabólitos celulares (SHARMA et al, 2018; AMINI, LAURENCIN, NUKAVARAPU, 2012).

Dispositivos tridimensionais, porosos, preparados com biopolímeros de origem natural, sintética ou compósitos, devendo ser biocompatíveis, biopersistentes e biodegradáveis, são comumente denominados *scaffolds* (LANGER, VACANTI, 1993). Os *scaffolds* destinados à regeneração do tecido ósseo devem apresentar características biomiméticas e fisiomecânicas compatíveis com o tecido nativo a ser reparado. Contudo, a regeneração tecidual por meio de scaffolds enfrenta desafios complexos. Para atingir sucesso, é necessário um profundo conhecimento anatômico, fisiológico, histológico e funcional do tecido ósseo e dos biomateriais utilizados (BARTOLD et al., 2000). Seu formato pode ser moldado conforme o local de aplicação; e sua estrutura final pode ser lamelar, laminada, cilíndrica, cúbica, granulada, entre outras. Desta forma, o projeto para a fabricação de *scaffolds* deve ser específico para cada região do corpo e cada tecido. Especificamente, além de assegurar a função estrutural, os biomateriais destinados à fabricação dos *scaffolds* devem influenciar o tecido hospedeiro para a liberação de fatores de crescimento relacionados a osteogênese e angiogênese. Embora muitos progressos tenham sido feitos, problemas cruciais na bioengenharia de tecidos dificultam o uso clínico dos *scaffolds* para regeneração de tecido ósseo. Nesse sentido, parece fundamental o desenvolvimento de biomateriais (*scaffolds*) que possam ser aplicados clinicamente e que aumentem a previsibilidade da neoformação do osso alveolar de suporte perdido em decorrência de exodontias ou de doenças inflamatórias como, as periodontites e periimplantites. No contexto específico da regeneração óssea, a associação de

diferentes biopolímeros pode ser uma estratégia eficaz para aprimorar as propriedades dos *scaffolds* (SAMPATH et al, 2018).

A quitosana (Qt) é um polissacarídeo derivado do processo de desacetilação da quitina, um biopolímero natural encontrado principalmente nas conchas de crustáceos marinhos e paredes celulares de fungos. Tem sido amplamente utilizada em uma variedade de campos biomédicos devido às suas características físico-químicas que facilitam a manipulação da estrutura molecular devido ao fato desta molécula ser pH-dependente. Por ser um material de baixo custo, natural, abundante, biodegradável e biocompatível, com propriedades atóxicas, mucoadesivas, hemostáticas e antimicrobianas, a Qt possui aplicações na engenharia de tecidos para a preparação de *scaffolds*, usados para regeneração óssea em odontologia e ortopedia (KHOR, LIM, 2003). No entanto, o uso de Qt em *scaffolds* é limitado, pois possui baixa bioatividade e baixas propriedades mecânicas, essenciais para a regeneração óssea (AGUILAR et al., 2019). Estudos mostram que a mistura de Qt com outros polímeros sintéticos ou naturais pode controlar fatores como porosidade e retenção de água, aumentar suas propriedades mecânicas, reduzir a taxa de biodegradação do *scaffold*, assim como aumentar sua bioatividade e biocompatibilidade (AGUILAR et al., 2019).

Outros polímeros naturais, como o colágeno tipo 1 (COL-1), proteína da matriz extracelular caracterizada por três cadeias polipeptídicas, que representa 90% da matéria orgânica do osso, também têm sido usados em *scaffolds* para engenharia de tecidos há décadas. Este biomaterial é versátil e possui excelentes propriedades biológicas, sendo considerado um indutor de efeitos positivos na adesão, proliferação e diferenciação celular (WANG et al., 2015), influenciando o comportamento celular por meio de interações mediadas por receptores (ZHANG et al., 2017), além de fornecer uma estrutura tridimensional porosa (WANG et al., 2015). No entanto, o uso de COL-1 sozinho em *scaffolds* tem desvantagens, incluindo baixa rigidez biomecânica e suscetibilidade à biodegradação, pois é um alvo para múltiplas enzimas colagenase expressas por várias células (RICO-LLANOS et al., 2021). Portanto, um *scaffold* baseado em COL-1 não teria as propriedades físicas e biomecânicas ideais para a engenharia de tecido ósseo, pois se degradaria antes do tempo necessário para a osteogênese, deixando de suportar a

proliferação e diferenciação celular. Consequentemente, o COL-1 é frequentemente usado em combinação com outros biomateriais, como o a Qt, para formar compósitos que aumentam as propriedades físicas e biomecânicas dos *scaffolds*, ao mesmo tempo em que conferem suas propriedades biológicas a eles (ZHANG et al., 2017; NIKOLOVA, CHAVALI, 2019).

O ácido hialurônico (AH) também é um biopolímero natural abundante na matriz extracelular, que desempenha papel importante em vários processos bioquímicos celulares. Sua estrutura molecular é semelhante aos glicosaminoglicanos (GAGs) presentes na cartilagem articular e óssea, criando um ambiente favorável para o desenvolvimento de osteoblastos (COLLINS; BIRKINSHAW, 2013), além disso, apresenta importante biocompatibilidade e sua taxa de biodegradação pode ser controlada pela adição de outros biopolímeros. A estabilidade do hidrogel de AH como material para fabricação de *scaffold* depende em grande parte da sua baixa densidade, e o mecanismo de formação de hidrogéis e a densidade da reticulação também orientam a forma pela qual células e moléculas são incorporadas e liberadas do interior do *scaffold*. O AH possui excelente capacidade de absorção de água, tornando-o viscoelástico, o que significa que exibe comportamento elástico e viscoso. Ele pode ser temporariamente deformado quando o estresse é aplicado, mas retorna rapidamente à sua forma original quando o estresse é removido. A viscoelasticidade é uma propriedade mecânica intrínseca do tecido natural e, portanto, é considerada importante em biomateriais usados em *scaffolds* projetados para engenharia de tecido ósseo e cartilaginoso (HUANG et al., 2019). Essa propriedade pode ser transferida para o *scaffold*, tornando o HA um material promissor para a regeneração tecidual, facilitando a adesão, proliferação e diferenciação celular.

Assim, o presente estudo teve o objetivo de avaliar a biocompatibilidade e as propriedades osteogênicas in vitro de um *scaffold* sintetizado a partir da associação de COL-1, Qt e AH.

2 ARTIGO

Bone matrix formation is facilitated by a dense lamellar scaffold composed of chitosan, collagen type I and hyaluronic acid.

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ABSTRACT

The goal of the present in vitro study was to assess the biocompatibility and osteogenic inducing properties of a new scaffold composed of chitosan, collagen type I, and hyaluronic acid. For in vitro assays, pre-osteoblastic immortalized cells were cultivated in standard medium (SD) and osteogenic medium (OM) in the following groups: a) Control (C) – cells cultured directly on the polystyrene plate, and b) Chitosan + Collagen type I + Hyaluronic Acid (CH + COL + HA) - cells cultured in a scaffold produced by the association of type I collagen, chitosan and hyaluronic acid. Cells carried in CH+COL+ HA scaffold presented similar metabolic activity compared to control group. After 14 days, CH+ COL+ HA scaffold induced a higher mineral nodule deposition compared to control group regardless of the cultured condition (SD or OM medium). In addition to, CH+COL+HA scaffold itself promoted an increase of alkaline phosphatase activity and mRNA levels for *Runx2* and *Ocn* genes, and these occurs earlier when compared to control group. Based on the results, it is possible to conclude that the dense lamellar scaffold composed of chitosan/collagen type I/hyaluronic acid stimulated osteogenic phenotype maturation of cells and can be a promising material for future bone regenerative approaches.

Keywords: biopolymers; collagen type I; chitosan; hialuronic acid; osteoblast; mineralization

INTRODUCTION

The use of dental implants is considered a viable and advantageous treatment option for the rehabilitation of totally or partially edentulous patients (1). Nevertheless, in some cases, the bone structure is not sufficient in terms of quality and volume for the implants to be installed satisfactorily and subsequent placement of the prostheses, making the rehabilitation with implants unfeasible. As a result, regenerative surgeries using bone grafts and their substitutes are often included in the treatment plan.

Among the possibilities of bone grafts available, the gold standard is the autogenous graft, which is an osseous graft harvested from a single anatomic site and transplanted to another site within the same individual. These grafts present a low risk of immunogenicity associated with osteoinductive and osteoconductive properties due to the fact that they conserve viable cells and growth factors necessary for osteogenesis. However, they have some disadvantages such as the need for an additional surgical area for graft removal, increased morbidity and post-surgical complications, and limited availability of bone to treat extensive bone defects (2,3). For that reason, there is an increasing interest in tissue engineering that includes the provision of cells, scaffolds, and signaling molecules (4) with the goal of expanding the indications of regenerative procedures. The scaffold may be considered as a three-dimensional, porous device prepared with natural and/or synthetic polymers, intended to supply structural support for cell attachment and growth, to guide the differentiation of stem cells, structurally aiding the newly formed tissue, and to restore anatomy and function similar to the lost bone tissue. Therefore, the scaffold structure must mimic the extracellular matrix (ECM) including glycosaminoglycans, glycoproteins, and glycolipids in order to promote the regeneration of target tissue, presenting similar properties to the autogenous bone graft but without its disadvantages. The polymers must be mechanically stable, biocompatible, bioactive, biopersistent, and biodegradable (5). However, even though different materials have been studied for several decades, the ideal biomaterial that could replace autogenous bone in a more predictable way is yet to be found.

Chitosan is a polysaccharide derived from the deacetylation process of chitin, a natural biopolymer primarily found in the shells of marine crustaceans and fungal cell walls. It is the second most important organic compound in nature after cellulose. As it is a low-cost, natural, abundant, biodegradable, and biocompatible material that has non-toxic, muco-adhesive, hemostatic, and antimicrobial properties, chitosan has pharmaceutical and biomedical applications (6), including tissue engineering in the preparation of scaffolds used for bone regeneration in both dentistry and orthopedics (7). Yet, the use of chitosan in scaffolds is limited since it has reduced bioactivity and mechanical properties, which are essential for bone regeneration (6). Studies show that blending chitosan with other synthetic or natural polymers can control factors such as porosity and water retention, increase their mechanical properties, reduce the scaffold's biodegradation rate, and enhance their bioactivity and biocompatibility (6).

Other natural polymers such as collagen type I, the most abundant protein in bone tissue, representing 90% of the bone's organic matter, have been also used in scaffolds for tissue engineering for decades. This biomaterial is versatile and has excellent biological properties, influencing cell behavior through receptor-mediated interactions (8) and leading to positive effects on cell adhesion, proliferation, and differentiation (9). However, using type I collagen alone in scaffolds has disadvantages, including low biomechanical stiffness and susceptibility to biodegradation, as it is a target for multiple collagenase enzymes expressed by various cells (10). Therefore, a collagen type 1-based scaffold would not have the ideal physical and biomechanical properties for bone tissue engineering, as it would degrade before the required time for osteogenesis, failing to support cell proliferation and differentiation. Consequently, collagen is often used in combination with other biomaterials, such as chitosan, to form composites that increase the physical and biomechanical properties of the scaffolds while conferring its biological properties to them (8; 11).

Hyaluronic acid is also a natural biopolymer abundant in the extracellular matrix, making it biocompatible and playing a key role in various cellular biochemical processes. Its molecular structure is similar to the glycosaminoglycans (GAGs) present in joint and bone cartilage, creating a

favorable environment for the development of osteoblasts (12). It facilitates cell adhesion, proliferation, and differentiation, contributing to tissue regeneration. Hyaluronic acid has excellent water absorption capability, making it viscoelastic, which means it shows both elastic and viscous behavior. It can be temporarily deformed when stress is applied but quickly returns to its original shape when the stress is removed. Viscoelasticity is an intrinsic mechanical property of natural tissue, and therefore, it is considered important in biomaterials used in scaffolds designed for bone and cartilage tissue engineering (13). This property can be transferred to the scaffold, making hyaluronic acid a promising material for tissue regeneration.

Blending two or more polymers with different properties to create a unique material with intermediate properties is an interesting approach to improve the properties of scaffolds (14). Chitosan has been combined with collagen primarily because collagen has a fast degradation rate in tissue regeneration applications, while chitosan has a much slower degradation rate compared to collagen. Additionally, collagen can efficiently control the porosity and water retention of chitosan-based scaffolds, enhance their bioactivity and biocompatibility, and increase their mechanical properties (6). When hyaluronic acid is added to the composition of a chitosan/collagen blend scaffold, there is an improvement in the scaffold's elasticity and resistance to deformation, leading to an increase in porosity. This may improve cell attachment to the scaffold surface and support cell migration to deeper layers, aiding cell growth and proliferation (15). Hyaluronic acid has affinity to both chondrocytes and osteoblasts, allowing it to be used in scaffolds for cartilage and bone repair (16).

Based on the properties of these biopolymers, the present study aimed to assess the biocompatibility and osteogenic-inducing properties of a scaffold composed of chitosan, collagen type I, and hyaluronic acid.

MATERIAL AND METHODS

Preparation of dense lamellar scaffold

Scaffold preparation was performed as described by Alves TRF et al., 2018, with some modifications to hyaluronic acid incorporation (17). Briefly, 3.0 g of the chitosan (CH; Sigma-Aldrich, MO, USA) was added into 100 mL of the

glacial acetic acid solution (1.5% v/v) and shaken until complete dissolution. The collagen dispersion was prepared by addition of 2 mL DMEM (Sigma-Aldrich, MO, USA), 1 g of collagen type I (COL; NovaProm Food Ingredients Ltda, SP, Brazil) and water enough to obtain 10 mL of dispersion. The COL and CH hydrogels were mixed in the rate of 9:1 (m/m) and this hydrogel was used to prepare the COL-CH scaffold. Then, 0.06 g of hyaluronic acid (AH; Sigma-Aldrich, MO, USA) was added and homogenized with 2 cycles and pressure of 700 bar. The dispersions were placed into cylindrical containers (inner diameter=21 mm and height=11 mm), and incubated at 10°C for 24 h for polymerization. Next, dense lamellar scaffold was produced by plastic compression, using hydrostatic press (4KN) for 10 min. Finally, the matrices were freeze-drie, resulting in cross-linked collagen-chitosan-hyaluronic acid scaffold.

Porosity, interconnectivity and pore size

The morphometric characteristic of the porosity, interconnectivity and pore size of scaffolds were evaluated by computerized microtomography (μCT). The scaffolds pictures were captured by X-Ray microtomograph (Brucker-micro CT-SkyScan 1174, Kontich, Belgium) with high resolution scanner (28 mM pixel and integration time at 1.7 s). The source of the X-rays was 34 keV of energy and 790 mA of current. The projections were acquired in a range of 180° with an angular step of 1° of rotation. 3D virtual models representative of various regions of scaffolds were created and the data were mathematically treated by CT Analyzer v. 1.13.5.

Scanning Electron Microscopy – SEM

SEM photographs scaffolds were obtained using a scanning electron microscope (LEO Electron Microscopy/Oxford, Leo 440i, Cambridge, England) with a 10 kV accelerating voltage. All samples were affixed to a brass specimen holder using double-sided adhesive tape, and the powders were made electrically conductive by coating with gold using a sputter-coater for 4 min at 15 mA. The images were obtained with tha magnification at 200 – 500X for descriptive analysis.

Cell culture and experimental groups

MC3T3-E1 cells of a murine bone calvaria pre-osteoblast (ATCC, VA, USA) were cultured in standard medium consisting of alpha MEM culture medium (Invitrogen, MA, USA) with 10% fetal bovine serum, 2% penicillin/streptomycin (Invitrogen, MA, USA), 0,5% gentamicin and 0,5% amphotericin B (Sigma-Aldrich Co, Saint Louis, USA), at 370C in a humidified atmosphere with 5% CO2 and 95% air. For the in vitro assays, the following groups were considered:

a) Control (C) – cells cultured directly on the polystyrene plate;

b) Chitosan + Collagen type I + Hyaluronic Acid (CH + COL + HA) - cells cultured in a scaffold produced by the association of type I collagen, chitosan and hyaluronic acid.

In each experimental assay, a concentration of 0,5 or 1,0 x 104 cells was plated into each scaffold, using a volume of 20 – 40 µl of α-MEM. The cells + scaffold sets were incubated at 37°C, in an atmosphere containing 95% air and 5% carbon dioxide (CO2) for 1 hour, to allow initial cell adhesion. Then, the scaffolds + cells were transferred to a new 96-well plate (Corning, NY, USA) and added 100 µl of α-MEM supplemented with10% fetal bovine serum, 2% penicillin/streptomycin (Invitrogen, MA,USA), 0,5% gentamicin and 0,5% amphotericin B (standard culture medium – SD Medium). All assays were performed in triplicate for each group and treatment, with only the scanning electron microscopy analysis performed in duplicate.

Cell metabolic activity assay

Cells were plated in 96-well plates in a concentration of $0{,}5x10^4$ cells/scaffold in a standard medium and incubated at 37°C, in an atmosphere containing 95% air and 5% carbon dioxide (CO2), with the culture medium being changed every three days. At the end of each period (days 1, 3 and 7), 20ul of CellTiter96®AQ_{ueous} One Solution Reagent – MTS assay (Promega, Madison, WI, USA) was added to each well and the cells incubated at 37°C in a humidified 5% CO_2 incubator. After 2 hours of incubation, the scaffolds were removed and the absorbance of the formed formazan dye was measured photometrically at a wavelength of 490 nm in the VersaMax ELISA Microplate Reader (Molecular Devices,CA, USA).

Mineralization assay

The alizarin red staining (AR-S) assay was performed to identify the ability of scaffold to induce mineralized matrix formation. For this, cells were plated in 96-well plates at a concentration of 0,5 x 10^4 cells/*scaffold* for the groups containing the scaffolds, and in 24-well plates at a concentration of 1,0 x 10^4 cells/well for the control group. To evaluate the potential for mineralized matrix formation, cells were cultured under two different conditions within each experimental group: A) SD medium: cells cultured in standard culture medium B) OM medium: cells cultured in osteogenic induction medium composed of α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 50 µg/mL ascorbic acid and 10 mM β-glycerolphosphate (Merck, Germany). The cells were kept under these culture conditions for a period of 14 days, with the culture medium being changed every 3 days. At the end of 14 days, the samples were fixed in ice-cold 70% ethanol for 1 hour and stained with 40 mM alizarin red solution (pH 4.2) at room temperature for 10 minutes under stirring. Then, aiming to compare the mineralization potential between the different experimental groups, the decolorization procedure of the nodules was performed for the quantitative analysis. For this, 1 mL of a 10% cetylperidine chloride solution (pH 7.0) was added for 15 minutes and the aliquots of this solution were subjected to absorbance reading at 562 nm in the VersaMax ELISA Microplate Reader (Molecular Devices,CA, USA). Since the *scaffolds* are porous, favoring the incorporation of the alizarin red dye, samples of the biomaterials were kept in a standard culture medium for 14 days without cells, under the same conditions as the experimental groups, and were also submitted to the AR- S. The values of the absorbance reading of the sacaffold samples were discounted from the readings of the test groups (*scaffold* + cells), with the objective of obtaining a final absorbance value that corresponded to the color of the mineralized nodules formed by MC3T3 cells inside the scaffold.

Quantification of Alkaline Phosphatase Enzyme (ALP) Activity

Cells were plated at a concentration of 0,5 x 10^4 cells/*scaffold* in 96-well plates, and cultured in standard culture medium (SD medium) or in osteogenic medium (OM medium) for 4, 7 and 10 days, to quantify the activity of the alkaline phosphatase enzyme. At the end of each period, ALP activity was

analyzed using a commercially available kit (1-Step™ PNPP Substrate Solution, Thermo Scientific), following the manufacturer's recommendations. Briefly, cells were lysed with Tris buffer (0.5M – pH 9.0), NaCl (0.9%) and Triton X-100 (1%), centrifuged (12,000 xg, 15 min, 4°C) and the supernatant incubated with 1-Step PNPP substrate (200µl/sample) for 30 minutes at room temperature and protected from light. Then, 50 ul of 2N NaOH was added to stop the reaction and the absorbance reading was performed at 405 nm in the VersaMax ELISA Microplate Reader device (Molecular Devices, Sunnyvale, CA, USA), with the absorbance corresponding to the amount of the alkaline phosphatase enzyme produced by cells.

Gene expression analysis – Real-time polymerase chain reaction (RT-qPCR)

Cell maturation into an osteoblastic phenotype was confirmed by analyzing gene expression for the markers *Runx2* (Runt-related transcription factor) and *Ocn* (osteocalcin). For this, cells were plated at a concentration of 0,5 x 10^4 cells/*scaffold* in 96-well plates, and cultured in standard culture medium (SD medium) or in osteogenic medium (OM medium) for 10 and 14 days. At the end of each period, total RNA was extracted using the TRIzol reagent (Invitrogen™, MA, USA), following the manufacturer's recommendations. Then, the RNA samples were suspended in RNase-free MiliQ water and stored at -70°C. A 1µl aliquot (1:100 dilution) was used to obtain the RNA concentration of the samples, using a spectrophotometer (Nanodrop 2000, Thermo Scientific). Total RNA was treated to eliminate any DNA residue in the sample (DNA-free™, Ambion Inc.,TX, USA), and 1µg of the sample was used for the synthesis of complementary DNA (cDNA). For this, the reactions were performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA), as recommended by the manufacturer for a final volume of 20µl.

The primers for *Gusb* and *18S* (reference genes), *Runx2* and *Ocn* were designed with the aid of a program specifically developed to design primers for the LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) (Table 1). The efficiency of the reactions for each primer was optimized prior to the start of the actual qPCR reactions. Real-time PCR reactions were

performed with the LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) using the LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics GmbH, Mannheim, Germany). The reaction profile was determined following the formula suggested by the equipment manufacturer. For each of the runs, water was used as a negative control and the reaction product was quantified using LightCycler Relative Quantification Software (Roche Diagnostics GmbH, Mannheim, Germany). The RT-qPCR reactions for the reference genes were analyzed using the Normfinder software, which identified the *18S* gene as the most efficient for normalizing target gene reactions.

Statistical analysis

All experiments were performed in triplicate with at least one repetition of each, performed at different times. Data were analyzed for normality using the Kolmogorov-Smirnov test and were expressed as mean and standard deviation. Student's t test was used for analysis between two groups and the One-way ANOVA test followed by multiple paired comparison (Tukey's test) was used to identify differences between three or more groups. Statistical analysis was performed using the GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA), considering a value of $\alpha = 0.05$.

RESULTS

Characterization of dense lamellar scaffold

The morphological and morphometric characteristic of dense lamellar scaffolds are showed in Figure 1 and Table 2. The CH-COL-HA scaffolds showed 4 cm diameter, and flexible structure when hydrated with saline solution (Fig. 1A and 1B). The scaffolds had the regular interconnected structure with large porosity (Table 1). The pores interconnectivity of the CH-COL-HA scaffolds were 75.23% (Table 2), and the pores were oriented with round shape as observed by SEM (Fig. 2A and 2B).

Cell metabolic activity assay

The metabolic activity of MC3T3 cells carried on dense lamellar scaffold was compared to control group at the following time points: 1, 3, and 7 days,

using MTS assay. As shown in Figure 3, CH+COL+HA did not affect the metabolic activity of MC3T3 cells, and there was no difference between this group compared to control. This finding suggest that MC3T3 cells maintain the proliferation capacity when cultured on dense lamellar scaffold.

Dense lamellar scaffold induced mineralized matrix deposition by MC3T3 cells

The ability of CH+COL+HA scaffold to induce mineralized matrix formation by MC3T3 cells was evaluated using alizarin red staining (AR-S). After 14 days in culture, cells seeded in CH+COL+HA scaffold showed a higher mineral nodule deposition compared to control group regardless of the cultured condition (SD or OM medium) (Figure 4A). This result shows that the scaffold alone is already able to induce mineralized matrix formation by pre-osteoblastic cells (MC3T3 cells).

Dense lamellar scaffold stimulates early expression of osteogenic markers by MC3T3 cells

To quantify the activity of the alkaline phosphatase enzyme (ALP), cells were cultured in SD and OM medium for 4, 7 and 10 days. Data analysis showed the presence of ALP activity in cells carried in the scaffolds in all periods (Figure 4B). On day 4, cells cultured in standard condition (SD medium), presented higher levels of ALP for CH+COL+HA group when compared to the control group (Figure 4B). Further, the osteogenic medium induction did not increase the levels of ALP in CH+COL+HA group, showing that the scaffold composition itself has already stimulated enzymatic activity. On the other hand, ALP activity in control group was only observed in cells cultured under osteogenic condition after 7 and 10 days (Figure 4B).

Next, to confirm the osteogenic induction potential of CH+COL+HA scaffold, RT-qPCR was performed to assess gene expression for *Runx2* and *Ocn*. At day 10, the results of RT-qPCR analysis revealed a significantly increase of *Runx2* levels transcripts for cells seeded in CH+COL+HA scaffold and cultured in SD medium (Figure 4C). At this time, it was also possible to observe a significant intragroup difference for scaffold group, with cells cultured in SD medium having a higher levels of *Runx2* transcripts than cells in OM

medium (Figure 4C). After 14 days, mRNA levels for Runx2 showed increased only in CH+COL+HA group under osteogenic condition.

The results for *Ocn* gene expression showed that cells carried in CH+COL+HA group had a higher levels of transcripts compared to control group in both periods (10 and 14 days) independently of culture condition (SD or OM medium) (Figure 4D). At day 10, it was found a significantly higher expression of *Ocn* gene in CH+COL+HA group under osteogenic condition. However, as described above, the scaffold composition itself was able to stimulate *Ocn* gene expression, since in the control group the transcripts for this gene were only found in OM medium with higher levels after 14 days (Figure 4D).

DISCUSSION

Regenerative surgeries involving bone grafts and biomaterials are often included in the treatment plan of patients requiring implant rehabilitation, particularly when there is inadequate bone structure in terms of quality and volume. Autogenous grafts are considered the gold standard for bone grafting; however, they come with certain drawbacks. These include the need for an additional surgical site for graft removal, increased morbidity and postoperative complications, and limitations in the availability of bone for extensive bone defects (19,2,3). Consequently, there is a demand for regenerative approaches that can improve osseous anatomy and quality while minimizing associated morbidity. Tissue engineering approaches, particularly the development of scaffolds, have gained significant interest in this regard. Scaffolds are three-dimensional porous structures prepared using natural and/or synthetic polymers, aiming to promote the regeneration of target tissues, offering comparable properties to autogenous bone grafts, but without their inherent disadvantages (5).

Scaffolds can be prepared using a single polymer or a blend of multiple polymers with distinct properties, aiming to create a unique material that combines the intermediate properties of the individual polymers. This approach enhances the scaffold's structure and overall properties (14). Previous studies have demonstrated the benefits of blending chitosan and type I collagen, two natural biopolymers, in scaffold preparation. This blend slows down the

degradation rate of collagen, improves the bioactivity and biocompatibility of chitosan, and enhances the mechanical properties of the scaffold (6,10). Additionally, the inclusion of hyaluronic acid in the blend has been shown to improve cell attachment to the scaffold surface, facilitate cell migration to deeper layers of the scaffold, and promote cell growth and proliferation (15). Based on these considerations, the present study investigated scaffolds composed of chitosan, type I collagen, and hyaluronic acid to evaluate their biocompatibility and osteogenic-inducing properties by performing a series of experiments using immortalized pre-osteoblasts (MC3T3-E1 cells) for cell culture.

Cell viability was assessed using MTS analysis after 1, 3, and 5 days by comparing cells plated directly in 96-well plates with cells plated in the scaffolds. The results demonstrated that cells plated in the scaffolds exhibited metabolic activity, as they were able to reduce the tetrazolium reagent into formazan, indicating their viability was not affected by the presence of the scaffolds. This confirms the non-toxicity and biocompatibility of the scaffold's composition, which is consistent with previous studies highlighting the biocompatibility of chitosan, collagen type 1, and hyaluronic acid, all of which have essential biological properties to provide bone regeneration, and therefore could be used in scaffolds (20, 21). Their beneficial biological properties explains why different blends using these polymers were made and analyzed throughout the years, and aligns with the choice of these polymers for the composition of the scaffold analyzed in the present study. However, the MTS analysis revealed that the metabolic activity of the cells remained constant throughout the 5-day experiment, indicating a lack of cell proliferation. This result is consistent with the scanning electron microscopy images obtained after 24, 48, and 72 hours of cell loading, showing isolated cells adhered to the scaffold surfaces, suggesting that the cells were still in the adhesion phase rather than actively proliferating on the biomaterial. Based on these observations, it can be suggested that the scaffolds, while biologically compatible, presented a more challenging surface for MC3T3-E1 cell adhesion and proliferation. This difficulty may be attributed to the scaffold's structure, which can directly influence cell behavior, including factors such as porosity and pore size (22). Thus, these findings highlight the

need to optimize the scaffold's design to promote enhanced cell adhesion, proliferation, and tissue regeneration.

Porosity is a critical characteristic that influences cell accommodation within the scaffold, promoting favorable scaffold-cell interactions and facilitating efficient transport of nutrients throughout the three-dimensional matrix. It enables cells to adhere to the scaffold surface, migrate to deeper layers, and support their growth and proliferation (23; 24; 25; 15). The level of porosity, along with pore size, significantly impacts cell adhesion and proliferation within the scaffold (26), since the network structure of pores guides and promotes the formation of new tissue, as demonstrated in various studies (27, 28). However, caution must be exercised as increased porosity and large pores can compromise the scaffold's mechanical properties, leading to potential degradation (29). Sionkowska et al.,2016, investigated the impact of hyaluronic acid addition to a chitosan/collagen scaffold and observed increased scaffold elasticity and porosity. This enhancement facilitated cell attachment, migration to deeper layers, and proliferation (15). While the exact porosity measure of the scaffold in this study remains unknown, augmenting the hyaluronic acid content, which is currently 1%, could potentially increase porosity. It is recommended to repeat the viability assay and electron microscopy analysis after changing the scaffold's composition to assess cell viability and observe cells in the proliferation phase, not solely the adherence phase. Pore sizes in scaffolds are typically categorized into macropores and micropores, and each range has different effects on cell behavior and tissue regeneration (30). Macropores refers to relatively large pore sizes, which provide ample space within the scaffold for cell colonization, tissue ingrowth, and vascularization, being essential for accommodating cells and facilitating the formation of blood vessels and new tissues (31). On the other hand, micropores refer to the presence of small pore sizes, influencing protein adsorption, cell anchorage, and overall cell behavior, contributing to successful bone tissue regeneration (32). The ideal pore sizes for bone tissue engineering are still subject to ongoing research and debate. Studies have suggested different optimal pore size ranges depending on specific factors such as cell type and the stage of tissue regeneration. For example, optimal pore sizes for osteoblast proliferation have been reported by Lim et al., 2004, to be in the range of 200-350 µm, while

pore sizes of 500 µm did not significantly affect cell attachment (33). Moreover, O'Brien et al., 2004, suggested the ideal pore size for initial cell adhesion *in vitro* would be 95µm (34), while Murphy et al., 2010, reported that the optimal range for in vitro bone engineering pore sizes would be 100-325µm (22). When designing and fabricating scaffolds, it is important to strike a balance between porosity and mechanical properties. The final porosity and pore sizes should be considered in order to provide a suitable microenvironment for cell behavior, nutrient transport, and tissue regeneration (26). Unfortunately, the porosity and pore size of the scaffold in the present study are unknown, highlighting the importance of obtaining these measures to better understand their influence on the obtained results.

The osteogenic induction potential of the scaffold was demonstrated with the results of the Alizarin Red staining assay. After 14 days in culture, an increase in the deposition of mineral nodules was observed in the scaffold group compared to the control group, regardless of the culture medium used. This suggests that the biomaterial alone was capable of inducing the maturation of MC3T3-E1 cells into an osteoblastic phenotype, even without specific osteogenic stimulation. This finding confirms the osteoconductive capacity of the scaffold, which refers to its ability to provide a suitable environment for the infiltration and activity of undifferentiated mesenchymal cells, osteoblasts, and osteoclasts. Additionally, the scaffold exhibited osteoinductive capacity, promoting the multiplication and differentiation of undifferentiated mesenchymal cells into osteoblasts.

These properties are crucial for the process of bone regeneration. The positive effects of the scaffold may be attributed to the polymers used in the scaffold's composition. Chitosan, in particular, has been reported to stimulate cells to produce and release cytokines, which play a role in promoting osteogenesis and angiogenesis (35), and to influence both cell activity and mineralization, making it a promising material for tissue engineering applications (36). A review by Aguilar et al., 2019, highlighted this enhanced property of chitosan when combined with other polymers or bioactive molecules (6). The review suggested that chitosan-based biocomposite scaffolds have the potential to promote stem cell proliferation and differentiation, accelerate tissue regeneration, and facilitate angiogenesis and vascularization. Promising results

have been observed in various animal models, indicating the potential future application of these chitosan-based biocomposite scaffolds in human clinical trials. Overall, the results confirm the osteogenic potential of the scaffold and its ability to induce the maturation of MC3T3-E1 cells into osteoblasts. The composition of the scaffold, particularly the inclusion of chitosan, contributes to its positive effects on cell activity, mineralization, and the potential for tissue regeneration.

The mineralization of bone tissue is a complex process that involves the controlled deposition of calcium and inorganic phosphate, leading to the formation of hydroxyapatite crystals in the extracellular matrix. This process is crucial for the structural integrity and strength of bone tissue (37, 38). During biomineralization, the deposition of a collagenous extracellular matrix is essential. This matrix serves as a substrate for mineral formation, and there is evidence suggesting that collagen plays a role in directing the nucleation process of hydroxyapatite crystals (39, 40). Alkaline phosphatase (ALP) is an enzyme that plays a significant role in this mineralization process. It is produced by various cells involved in bone formation, including osteoblasts, which are responsible for synthesizing and depositing the organic matrix of bone (41). ALP's enzymatic activity contributes to the regulation of mineralization through several mechanisms. First, it plays a crucial role in regulating the balance between promoter molecules (Pi-phosphate) and inhibitor molecules (pyrophosphate-PPi) during the mineralization process (42), being responsible for the degradation of pyrophosphate (PPi) into inorganic phosphate (Pi) in the extracellular environment. In that way, it promotes the availability of phosphate ions by hydrolyzing organic phosphate compounds, such as inorganic pyrophosphate, which acts as an inhibitor of mineralization (42). By breaking down these inhibitors, ALP increases the concentration of free phosphate ions available for mineralization (42). Secondly, ALP participates in collagen maturation. Collagen molecules undergo post-translational modifications, including the addition of phosphate groups, to form mature collagen fibrils. ALP plays a role in this maturation process by dephosphorylating specific amino acids in collagen, allowing for proper cross-linking and maturation of the collagen fibrils. Therefore, the presence and activity of ALP are essential for

proper bone mineralization, ensuring the formation of structurally sound and mineralized bone tissue (43).

The results of the ALP assay revealed that the scaffold composition has the potential to stimulate enzymatic activity associated with osteoblastic differentiation and mineralized matrix formation. Initially, there was an increase in ALP activity in the scaffold group cultured in standard medium compared to the control group cultured under the same condition, suggesting the influence of the scaffold on osteoblastic phenotype maturation and early stages of mineralized matrix formation. However, over time, the ALP activity in the scaffold group decreased compared to the control group, in both culture mediums, indicating a reduction in the maintenance of ALP activity by the scaffold. This indicates a discrepancy between the ALP assay results and those obtained from the Alizarin Red assay. In contrast to the ALP assay, the Alizarin Red assay demonstrated that the scaffold group exhibited a higher deposition of mineral nodules compared to the control group after 14 days in culture, regardless of the culture medium used. One possible explanation for this finding would be an increase in the expression of the enzyme NPP1 (nucleotide pyrophosphohydrolase-1), which produces extracellular pyrophosphate (PPi), acting as an ALP antagonist. Increased NPP1 expression leads to the conversion of inorganic phosphate (Pi) into extracellular pyrophosphate (PPi), inhibiting the nucleation of hydroxyapatite crystals (42, 44). Both ALP and NPP1 enzymes play a role in maintaining the balance between mineralization promoters and inhibitors,crucial for proper bone formation and remodeling (45). Hence, the significant increase in ALP activity observed in the scaffold group may have been associated with a negative feedback mechanism process through increased NPP1 expression. To validate this hypothesis, future analyses should focus on examining the expression levels of the NPP1 enzyme and its corresponding gene, ENPP1. Overall, the results indicate that although the ALP activity may not have remained consistently elevated, the scaffold facilitated an increased deposition of mineral nodules compared to the control group, as demonstrated by the Alizarin Red assay. These results support the scaffold's osteogenic potential and suggest its capability to promote mineralization in the context of bone tissue engineering. However, this discrepancy suggests the presence of limitations or factors that influenced the

actual mineralization process in the scaffold group. Further investigation and potential modifications are necessary to enhance mineralization in future experiments.

Confirmation of cell maturation into an osteoblastic phenotype was achieved through the analysis of genes associated with osteoblastic differentiation and bone formation: *Runx2* (Runt-related transcription factor) and *Ocn* (osteocalcin). These genes are associated with osteoblastic differentiation and bone formation. *Runx2* is a critical transcription factor involved in regulating osteoblast differentiation and bone formation, and increased expression of *Runx2* indicates the activation of osteogenic pathways and commitment to an osteoblastic phenotype for promoting bone formation (46, 47). *Ocn*, on the other hand, serves as an important marker for mature osteoblasts and is involved in the regulation of bone mineralization (48). The increased expression of both *Runx2* and *Ocn* in response to the scaffold material confirms its osteogenic potential (49). MC3T3-E1 cells, being pre-osteoblastic in nature, possess the genetic machinery required for osteoblastic differentiation, including a basal expression of both *Runx2* and *Ocn*. However, the increase in the expression of these genes typically occurs after a period of cell proliferation, which can vary between 4-10 days in MC3T3-E1 cells depending on the specific culture conditions (50). Therefore, due to the period of cell proliferation and the potentially challenging conditions of the scaffold surfaces for cell growth, the gene expression analyses for *Runx2* and *Ocn* were performed for a duration of 10 and 14 days. The results indicate that the presence of the scaffold material, especially when combined with osteogenic factors in the culture medium, has a positive impact on the expression of both *Runx2* and *Ocn* genes. The differences observed between the Scaffold and Control groups, as well as between different culture conditions within each group, suggest that the scaffold material itself influences gene expression, promoting osteogenic differentiation. These findings support the potential of the scaffold to influence gene expression, enhance osteoblastic activity and promote bone tissue regeneration, highlighting the material's osteogenic properties; and align with previous research highlighting the osteogenic potential of scaffold materials, particularly those incorporating chitosan, collagen, and hyaluronic acid. Studies such as Sultankulov et al.,2019, have demonstrated the ability of these scaffold

materials to influence osteogenic gene expression and promote bone formation (21). The combination of chitosan, collagen, and hyaluronic acid in scaffold compositions has been reported to create a favorable environment for osteoblastic activity and mineralization, as described by Aguilar et al., 2019 (6). The observed upregulation of osteogenic markers in the gene expression analysis and the successful formation of mineralized nodules detected in the Alizarin Red assay further support the literature, confirming the scaffold's ability to enhance osteoblastic differentiation and facilitate bone tissue regeneration.

The combined findings from the gene expression analysis, Alizarin Red assay, and MTS assay provide a comprehensive understanding of the scaffold material's osteogenic potential and functionality. The MTS assay confirms cell viability and metabolic activity within the scaffolds, although the constant metabolic activity during the assay and presence of isolated cells in the scanning electron microscopy images suggest limited cell proliferation. These results emphasize the importance of perfecting the scaffold's design to enhance cell adhesion, proliferation, and tissue regeneration. Moreover, the increased expression of osteogenic markers observed in the gene expression analysis, along with the formation of mineralized nodules detected in the Alizarin Red assay, indicate successful osteoblastic differentiation and mineralization induced by the scaffold material. The increased deposition of mineral nodules in the scaffold group compared to the control group further supports these findings. Collectively, the results from these different assays demonstrate the scaffold's potential for promoting osteogenic differentiation, mineralization, and cell viability, while highlighting the need for further improvements to enhance cell proliferation and tissue regeneration.

CONCLUSION

It is possible to conclude that the dense lamellar scaffold composed of chitosan/collagen type I/hyaluronic acid stimulated osteogenic phenotype maturation of MC3T3 cells, and can be a promising material for future bone regenerative approaches.

Authors contribution

G.C.B.S. investigation, data curation, writing the original draft; C.M.S. methodology, formal analysis, investigation, data curation; N.A. conceptualization, methodology, funding aquisition, project administration; A.F.J. conceptualization, methodology, writing the original draft; D.G. conceptualization, methodology; M.V.C. conceptualization, methodology, formal analysis, investigation, data curation, writing the original draft; K.G.S. conceptualization, methodology, writing the original draft, supervision.

Declaration of competing interest

The authors declare no conflict of interest.

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Tables

Table 1. Primer sequences used for real-time quantitative PCR amplifications

Gene	Primer (5´ - 3´sequence)	Annealing temperature	Product Size
18S	F: CGTCTGCCCTATCAACTTTCG R: TGCCTTCCTTGGATGTGGTA	58ºC	124 pb
Gusb	F: TCTGAGGTGGAGGCGGAG R: GTAGGCGTAGATGGAGAGCA	60ºC	150 pb
Runx2	F: GAGGGAAGAGAGCAAGGGG R: GTGGTTGTTTGTGAGGCGAA	58ºC	180 pb
Ocn	F: CAGTCCCCAGCCCAGATC R: GCGTTTGTAGGCGGTCTTC	58ºC	135 pb

Actb: β-actin; Gapdh: Glyceraldehyde 3-Phosphate Dehydrogenase; Runx2: runt-related
transcription factor 2; Ocn: osteocalcin

Table 2. Morphological characteristics of CH-COL-HA scaffold

	CH-COL-HA dense lamellar scaffold
Volume of open pores (mm^3)	4.65
Pore interconnectivity (%)	75.23
Closed porosity (%)	75.26
Open porosity (%)	0.14

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Figure legends

Figure 1. Macroscopic characterization of CH+COL+HA dense lamellar scaffold before (A) and after hydration with saline solution (B).

Figure 2. Scanning electron microscopy of CH+COL+HA dense lamellar scaffold. (A,B) Representative image showing pores with rounded shape (X200 and X500 magnification, respectively). (C) Image at X200 magnification showing the scaffold surface.

Figure 3. The metabolic activity of MC3T3 cells was assessed by the MTS assay after periods of 1, 3 and 7 days in culture. There was no statistically significant difference between groups (Control *versus* CH+COL+HA), identified by the t test ($p < 0.05$).

Figure 4. Dense lamellar scaffold induces MC3T3 cells maturation into osteogenic phenotype. (A) Quantification of Alizarin Red (AR-S) identifying mineralized matrix deposition by MC3T3-E1 cells carried in dense lamellar scaffold. (B) Quantification of Alkaline Phosphatase Activity (ALP) at 4, 7, and 10 days. (C, D) mRNA levels for runt-related transcription factor 2 (Runx2) and osteocalcin (Ocn), respectively, assessed by qRT-PCR at 10 and 14 days. The experiments were performed in triplicate three times with comparable results obtained on each occasion The bars represent mean ± standard deviation. * Represents a statistically significant intragroup difference (SD versus OM) and # represents significant intergroup difference (Control versus CH+COL+AH), identified by the t test ($p < 0.05$).

3 CONCLUSÃO

Dentro dos parâmetros deste estudo, é possível concluir que o *scaffold* testado apresenta potencial como um material promissor para futuras abordagens na regeneração óssea. No entanto, é necessário aprimorar suas características para favorecer a adesão e proliferação celular.

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ANEXO 1 - Verificação de Originalidade e Prevenção de Plágio

DESENVOLVIMENTO DE SCAFFOLD LAMELAR DENSO À BASE DE QUITOSANA, COLÁGENO TIPO 1 E ÁCIDO HIALURÔNICO PARA FORMAÇÃO DE MATRIZ ÓSSEA

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ANEXO 2 – Comprovante de Submissão do Artigo

14/11/2023, 01:22 Fwd: Confirm co-authorship of submission to Carbohydrate Polymers - gabicbsallum@gmail.com - Gmail

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De: **Carbohydrate Polymers** <em@editorialmanager.com>
Date: qui., 9 de nov. de 2023 às 11:47
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Journal: Carbohydrate Polymers
Title: Bone matrix formation is facilitated by a dense lamellar scaffold composed of chitosan, collagen type I and hyaluronic acid.
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Co-Authors: Gabrielle Christine Bonetti Sallum; Catharina Marques Sacramento; Denise Grotto; Marco Vinícius Chaud; Norberto Aranha; Karina Gonzales Silvério
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