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Bone regeneration by a bone substitute biomaterial containing hydroxyapatite, chitosan, xanthan and graphene oxide supplemented with conditioned medium from mesenchymal stem cells.

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ABSTRACT

This study analyzed a recently developed bone substitute biomaterial made of chitosan-xanthan-hydroxyapatite-graphene oxide (CXHAG). The CXHAG particles underwent in vitro structural and morphological characterization, and in vivo testing with or without osteogenic conditioned medium from mesenchymal stem cells. ***Aim:*** *The aim of this study was to determine whether the CXHAG novel biomaterial, supplemented with conditioned medium from mesenchymal stem cells, could be useful for bone regeneration.* ***Materials and Method:*** *For the in vitro study, cells were incubated with 20mg of CXHAG granules for 24 hours and a MTT assay was performed to tests for cytotoxicity. For the in vivo study, critical size calvarial bone defects were created in twenty-five rats. One animal had the defect unfilled (Control Group–CG) and was euthanized after 42 days. Twelve rats received the CXHAG particles (Group 1–G1) and the other twelve received the CXHAG particles supplemented with the conditioned medium (Group 2–G2). All G1/G2 grafts were covered with a CXHAG membrane. G1/G2 animals were euthanized after 14 days (T1) or 42 days (T2). The specimens were processed and histologically evaluated.* ***Results:*** *SEM analysis of the CXHAG particles showed granules of 300-400µm, with a rough irregular surface. They were not cytotoxic to dental pulp stem cells in vitro. The CG specimen showed loose immature connective tissue and no bone formation at the center of the defect. G1 and G2 presented remnant biomaterial particles at both time points, but only G2 had bone formation at the center of the defect.* ***Conclusions:*** *The conditioned medium had a positive effect on bone regeneration in rat calvarial critical size defects when associated with the novel bone substitute biomaterial.*

Keywords: *bone regeneration - stem cells - chitosan - xanthan - graphene - conditioned medium.*

Regeneração óssea por meio de biomaterial substituto ósseo contendo hidroxiapatita, quitosana, xantana e óxido de grafeno suplementado com meio condicionado de células-tronco mesenquimais

RESUMO

Este estudo analisou um biomaterial substituto ósseo recentemente desenvolvido feito de óxido de quitosana-xantana-hidroxiapatita-grafeno (CXHAG). As partículas CXHAG observaram caracterização estrutural e morfológica in vitro. Foi testado in vivo, com ou sem meio condicionado osteogênico de células-tronco mesenquimais. ***Objetivo:*** *O objetivo deste estudo foi determinar se o novo biomaterial CXHAG, suplementado com meio condicionado de células-tronco mesenquimais, poderia ser útil para a regeneração óssea.* ***Materiais e Método:*** *Para o estudo in vitro, as células foram incubadas com 20mg de grânulos de CXHAG por 24 horas e foi realizado ensaio de MTT para verificar a citotoxicidade. Para o estudo in vivo, foram criados defeitos ósseos de tamanho crítico na calvária em vinte e cinco ratos. Um animal teve o defeito não preenchido (Grupo Controle – GC) e foi eutanasiado após 42 dias. Doze ratos receberam as partículas CXHAG (Grupo 1 – G1) e os outros doze receberam as partículas CXHAG suplementadas com o meio condicionado (Grupo 2 – G2). Todos os enxertos G1/G2 foram cobertos com membrana CXHAG. Os animais do G1/G2 foram eutanasiados após 14 dias (T1) ou 42 dias (T2). Os espécimes foram processados e avaliados histologicamente.* ***Resultados:*** *A análise SEM das partículas CXHAG mostrou grânulos de 300-400µm, com superfície áspera e irregular. Eles não foram citotóxicos para células-tronco da polpa dentária in vitro. As amostras CG mostraram tecido conjuntivo imaturo frouxo e nenhuma formação óssea no centro do defeito. G1 e G2 apresentaram partículas remanescentes de biomateriais em ambos os momentos, mas apenas G2 apresentou formação óssea no centro do defeito.* ***Conclusões:*** *O meio condicionado teve repercussões positivas na regeneração óssea em defeitos críticos de calvária de ratos quando associado ao novo biomaterial substituto ósseo.*

Palavras-chave: *regeneração óssea - células tronco - quitosana - xantana - grafeno - meio condicionado.*

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INTRODUCTION

The management of bone defects remains a major clinical challenge for both orthopedics and maxillofacial surgery. Guided bone regeneration (GBR) is commonly used to treat bone defects. The GBR technique uses a membrane as a barrier to exclude the proliferation of epithelial cells and connective tissue[1]. Because membranes can mimic the extracellular matrix, they serve as a support for cell growth, enabling proliferation and differentiation of specific tissues[2]. The principle of using membranes to select cell groups to populate the wound has been called osteopromotion and is usually used in conjunction with bone grafts[3].

Bone grafts used in GBR procedures can be natural or synthetic. They are classified according to source as autogenous (from the same individual), allogeneic (from another individual of the same species), xenogeneic (from an individual of another species) or alloplastic (synthetic materials)[4]. The association of synthetic ceramic derivatives with materials from natural sources seems to be a feasible alternative, since each type of material can contribute its advantages. Associations of ceramic derivatives and biopolymers such as chitosan/xanthan are gaining attention because in addition to being biocompatible and biodegradable, they interact with bone growth factors and receptor proteins[5]. Hydroxyapatite is a bioceramic that is often used for bone reconstruction purposes due to its striking resemblance to bone in terms of structure and characteristics[6]. Moreover, hydroxyapatite can overcome important issues concerning polymers, such as unfavorable mechanical characteristics regarding insufficient tensile and compressive strength. However, although its low resorption rate is related to the advantage of volume maintenance in bone reconstruction surgery, it also requires longer time for reconstruction[7]. Hydroxyapatite is therefore frequently employed in conjunction with various polymers and crosslinkers[6]. In addition, there are reports that biomaterials coated with graphene oxide can increase regenerative potential[8]. These materials can be combined with different polymers, ceramics and metals and, due to their ability to promote osteogenic differentiation, have been increasingly used to improve the physical, chemical and mechanical properties of biomaterials[9].

Critical-sized bone defects are defined as those that will not heal spontaneously within a patient's lifetime. The standard procedures to treat such defects are vascularized bone autografts, distraction osteogenesis, or tissue engineering. Autografts are considered the biological gold standard in the restoration of lost bone structure, due to their osteoinductive, osteoconductive and osteogenic properties. However, the tissue trauma caused when autogenous tissue is harvested has led to increased use of bone substitutes[10]. Nevertheless, bone substitute biomaterials are inadequate for treating critical bone defects because they are only osteoconductive[11]. Numerous recent articles have tested different methodologies[12-16] for adding osteoinductive and ultimately, osteogenic potential to bone substitutes.

Some studies suggest that mesenchymal stem cells or the conditioned culture medium in which they were grown (which contains proteins and growth factors) can improve bone regeneration associated with bone substitutes[17]. Although the use of mesenchymal stem cells has shown promise[18], it involves a major drawback: the need to introduce living cells into patients. For safety reasons, clinical use could be limited to autogenous applications. Even so, there is a concern that the stem cells might differentiate in unexpected ways or transform into a cancerous state.

Certain cell products can also promote tissue healing (e.g., bone cell proteins and growth factors) and may be useful for the treatment of critical size bone defects. Conditioned medium obtained from stem cell culture has been extensively studied in recent years[16,19-21]. The aim of the current study was to evaluate the use of a recently developed biomaterial composed of chitosan, xanthan, hydroxyapatite, and graphene oxide (CXHAG) in critical size bone defects. To potentially introduce osteoinductive properties to this novel biomaterial, a medium conditioned by stem cells was tested.

MATERIALS AND METHOD

Bone Substitute Biomaterial (chitosan-xanthan-hydroxyapatite-graphene oxide - CXHAG)

Graphene oxide (GO) was produced by liquid-phase exfoliation through the chemical route, using the Rourke et al. method[22]. The production of GO and its subsequent addition to the composite was based on the study by Lopes et al.[23]. First, 5 g of graphite flakes (GRAFLAKE 99550, National

Graphite) were suspended with 4.5 g of sodium nitrate ($NaNO_3$, VETEC) and 169 ml of sulfuric acid (H_2SO_4, HERZOG) under magnetic stirring for 2 h. Then, the mixture was cooled in ice, and 22.5 g of potassium permanganate ($KMnO_4$, VETEC) were slowly added and stirred for 2 h. The mixture was then left to stir for 7 days. The resulting mixture was slowly dispersed into 605 ml of 5 wt% H_2SO_4 for 1 h and stirred for a further 3 h. Hydrogen peroxide (16.5 g, 30 vol) was added to considerable effervescence and stirred for 2 h. The mixture was then further diluted with 500 ml of 3 wt% H_2SO_4/0.5 wt% H_2O_2 and left to stir overnight. After this period, the mixture was centrifuged (Hettich, model 420 R, at 9300 rpm, for 30–60 min, in 4 × 250 ml tubes), discarding the supernatant. This washing/centrifugation procedure was repeated 12 times using 500 ml of 3 wt% H_2SO_4/0.5 wt% H_2O_2 and 5 times using 500 ml of deionized water. Finally, vacuum filtration (EDWARDS, Germany) was applied to eliminate any non-oxidized graphite.

Chitosan-xanthan-hydroxyapatite-graphene oxide (CXHAG) was prepared in aqueous solutions rich in calcium and phosphorus precursors, with the addition of chitosan, xanthan, and graphene oxide[24]. The precursor solution was prepared by mixing, under stirring (magnetic stirrer – Quimis, São Paulo, Brazil), a solution of 2 mol/L lactic acid (Merck, Darmstadt, Germany), 1% (w/v) chitosan (Sigma-Aldrich, Saint Louis, USA), 0.5 mol/L calcium hydroxide (Merck, Darmstadt, Germany), 1% (w/w) graphene oxide in relation to the final hydroxyapatite, and a solution of 0.3 mol/L orthophosphoric acid (Merck, Darmstadt, Germany). After 24 hours of stirring, a solution of 1.2 mol/L potassium hydroxide (Sigma-Aldrich, Saint Louis, USA) and 1% (w/v) xanthan gum (Sigma-Aldrich, Saint Louis, USA) was added to adjust the pH to 12 at a temperature of 60 **°C,** enabling precipitation of the chitosan-xanthan-hydroxyapatite-graphene oxide composite.

To produce CXHAG granules, the powder produced was mixed with a 5% (w/v) pluronic aqueous solution and dried at 70 °C for 12 hours. The resulting composite was then ground using an agate mortar and pestle and transferred to a granulometric sieve with a mesh size between 300 and 400 µm **(**Bronzinox, 100 mesh and stainless-steel frame 5" x 2", São Paulo, Brazil) and sieved.

Membranes were produced following the method previously validated by Souza et al. (2022), which used the same materials (i.e., Chitosan/Xanthan membrane containing hydroxyapatite/Graphene oxide)[25].

Biomaterial Characterization

The crystal structure was investigated by X-ray diffraction, using a diffractometer (Malvern Panalytical, model X'Pert-MPD, Worcestershire, UK) on a Cu-Kα (λ=1.540 Å) tube, operating at 40 mA and 40 kV, and a scanning step of 0.02 seconds at 1 s/step. The HighScore Plus software was employed for qualitative analysis of the X-ray diffraction data.

The functional group was identified by Fourier Transform Infrared Spectroscopy (FTIR Prestige-21, Shimadzu, Columbia, USA) in the wavenumber range of 400-4000 cm^{-1}. Spectra were collected as the result of 32-64 scans with a resolution of 4 cm^{-1}. The analyses were performed at room temperature. For the selected spectra, the ratios of integrated intensities, and the integrated areas of the bands corresponding to O-H groups in the range 3470-3450 cm^{-1}, CO_3^{-2} groups in the range of 1380–1580 cm^{-1} and those due to PO_4^{-3} at 900–1300 cm^{-1} were calculated.

CXHAG particle morphology was investigated by scanning electron microscopy (LEO Electron Microscopy, model Leo 440i, Cambridge, UK), whereas energy dispersive spectroscopy (EDS) was used to qualitatively assess the chemical composition.

Lyophilized Conditioned Medium

Human dental pulp stem cells (catalog number PT-5025) obtained from Lonza (Lonza, Cohasset, USA) were used to prepare the lyophilized culture medium as previously described by Buss et al. (2023)[16]. Briefly, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Saint Louis, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, USA) and 1% antibiotic-antimycotic solution (Thermo Fischer/Life Technologies, Carlsbad, USA) and, after 24 hours, the medium was supplemented with 50 µM ascorbic acid, 10 mM ß-Glycerophosphate and 0.1 µM dexamethasone (Thermo Fischer/Life Technologies, Carlsbad, USA) to induce osteogenic differentiation. After 4 days, the conditioned medium was collected, frozen, and transferred to a lyophilizer (lyophilized at -55 °C under a vacuum of 0.040 m Bar for 48 h).

After lyophilization and sterilization by gamma radiation (Sterigenics, Americana, Brazil), the freeze-dried conditioned medium, in powder form, was dissolved in deionized water (20 mg/mL) and filtered through a 0.2 μm filter. Then, it was drip-associated with 100 mg of the biomaterial in 12-well cell culture plates (Corning, New York, USA) immediately before the surgery.

Cytotoxicity Assay of CXHAG Granules

For this study, dental pulp mesenchymal stem cells (Lonza, catalog number PT-5025, Cohasset, USA) were cultivated in 25 cm^2 cell culture flask using DMEM supplemented with 10% fetal bovine serum (FBS), 1% of antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) (Sigma-Aldrich, Saint Louis, USA), 2 mM L-glutamine (Sigma-Aldrich, Saint Louis, USA) and 100 μM ascorbic acid (Sigma-Aldrich, Saint Louis, USA). After incubation, at approximately 80% confluency, cells were detached using TrypLETM Express Enzyme (1x) (Gibco/ Life Technologies, Carlsbad, USA) at 37 °C for 3 min, and immediately seeded at a cell density of 1×10^4 cells into a 96-well culture plate, a final volume of 100μL/well. Experimental 20mg of CXHAG granules were also incubated in a 12-well plate. The cytotoxicity assay was performed in quadruplicate (n = 4), in accordance with ISO 10993-5 guidelines. After 24 h of incubation, 100 μL of the medium from the experimental pellets were transferred into a 96-well culture plate, 10 μL WST-1 solutions (Roche, Basel, Switzerland) were added to each well, and the cells were incubated at 37 °C in 5% CO_2 for 4 h. After the reaction period, the specimens were gently shaken for 1 min and the absorbance was measured at 450 nm by a microplate reader (Promega, Glomax E8032, Madison, USA).

The culture medium containing WST-1 without cells was used to set the background threshold, while culture medium containing WST-1 with cells was used as a control. As a cytotoxicity control, 50 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, USA) was used with 50 μL culture medium.

For data analysis of cytotoxicity assay, the Jamovi© statistical software (2.3.28.0 version) was utilized. The interaction between independent parameters was assessed using ANOVA repeated measures, followed by Tukey's post hoc test. A p-value of < 0.05 was considered statistically significant.

Experimental Design

Twenty-five eight-week-old male Wistar rats, weighing 300-350g, were used in this study, after approval from the Research Ethics Committee for Animal Experimentation of the Faculdade São Leopoldo Mandic (protocol no. 2020/010, approval date March 26th, 2020). The study was carried out in compliance with the ARRIVE guidelines.

One animal was enrolled in the control group (CG), in which nothing was grafted inside the bone defect, and euthanized after 42 days. The other twenty-four animals were randomly divided into two groups: Group 1 (G1) and Group 2 (G2). In G1 (n=12), bone defects were filled with the recently developed biomaterial and covered by the membrane. In G2 (n=12), bone defects were filled with the recently developed biomaterial combined with the conditioned medium and covered by the membrane. The animals in G1 and G2 were euthanized after 14 days (T1) or 42 days (T2).

Surgical Technique

After anesthesia, trichotomy in the region of the calvaria and subsequent antisepsis of the area, a 15 mm long linear incision was made with a scalpel in the integument covering the skull, followed by total flap detachment. The critical bony defect was made using an 8.0 mm diameter trephine drill (Maximus, Contagem, Brazil), crossing the entire bone thickness of the diploe. The bone fragment was removed, exposing the meninges at the bottom of the defect. Sequentially, the defect was filled with biomaterial according to the determined groups, covered with membranes measuring 10 mm x 10 mm (Fig. 1), and finally the flap was repositioned and sutured. The volume of the bone substitute biomaterial used in this study was determined by the aim to completely fill the bone defect.

After surgery, the animals received intraperitoneal postoperative medication for analgesia (Dipyrone 0.5g/mL, Algivet®-VETNIL, Louveira, Brazil).

Histologic Processing and Analysis

After 14 days or 42 days, according to the euthanasia time, the specimens were harvested and processed for histological evaluation. The calvarias were demineralized in 20% formic acid, dehydrated, and embedded in histological paraffin, to cut sections 4 μm thick in the central region of the defects. The sections were stained with hematoxylin-eosin

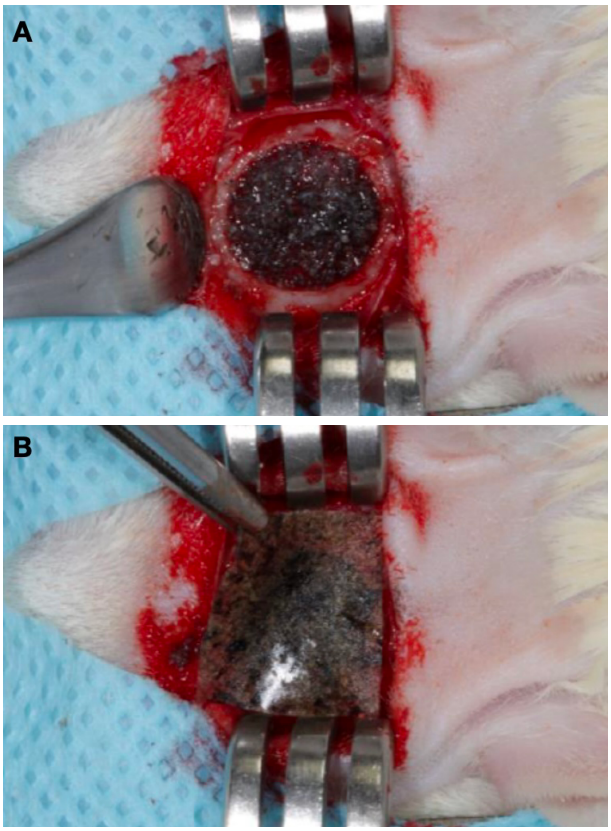

Fig. 1: Critical-size defect used in the study. A) Critical-size defect filled with the novel biomaterial, B) Defect and biomaterial covered with the membrane.

and mounted as photomicrographs on resin slides. Images were captured with a computerized imaging system (AxioVisionrel 4.8, Carl Zeiss, Oberkochen, Germany) coupled to the Axioskop 2 Plus light microscope (Carl Zeiss, Oberkochen, Germany).
Descriptive histological analyses were performed in the center of the defect. For bone formation at the center of the defect, the area of newly formed bone was traced using ImageJ software (National Institutes of Health, Bethesda, USA) on photomicrographs taken at 200x magnification. Photomicrographs were taken under a light microscope using a computerized image analysis system consisting of an Axioskop 2 plus light microscope (Carl Zeiss, Gottingen, Germany) connected to a microcomputer using AxioVision rel. 4.8 image analysis software (Carl Zeiss, Gottingen, Germany). The results were scored in square micrometers and then expressed as a percentage of the total area.

RESULTS

Analysis of the novel bone substitute biomaterial

Fig. 2A presents the XRD analysis of the CXHAG

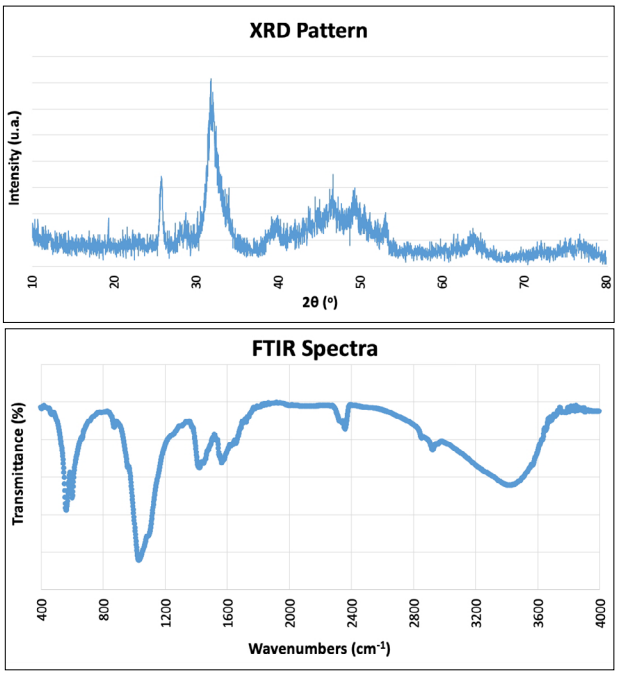

Fig. 2: A) XRD Patterns and B) FTIR Spectra.

granules, revealing patterns indicative of the hydroxyapatite phase, as aligned with JCPDS 09-0432 hydroxyapatite crystallographic record. Fig. 2B displays the spectra for the CXHAG granules, exhibiting bands at approximately 1,095, 1,045, 962, 607 and 577 cm^{-1}, which correspond to phosphate groups. The hydroxyl band at around 3,570 cm^{-1} is not distinctly defined in the granules. Moreover, minor peaks associated with the C-O vibrational bands of carbonate groups are evident within the 1,410-1,490 cm^{-1} range. Additionally, the CXHAG granules exhibit a distinct band for amino groups (C–CH_3), denoting the presence of chitosan, alongside a characteristic band for carboxylic groups (CO_3) from xanthan gum.
Fig. 3 shows that the granulation technology produced irregularly shaped granules with sharp edges. The granule surfaces at the micro level are rough. By measuring the granule dimensions from the SEM images, the value of the experimental granule size was determined to be between 300 and 400 μm.

In vitro assessment

Fig. 4 shows the *in vitro* cytotoxicity assay graph. Through the values for the viability percentage, it was observed that after an exposure of 24 hours to dental pulp mesenchymal stem cells (hDPSCs), no cytotoxicity was observed in the CXHAG group.

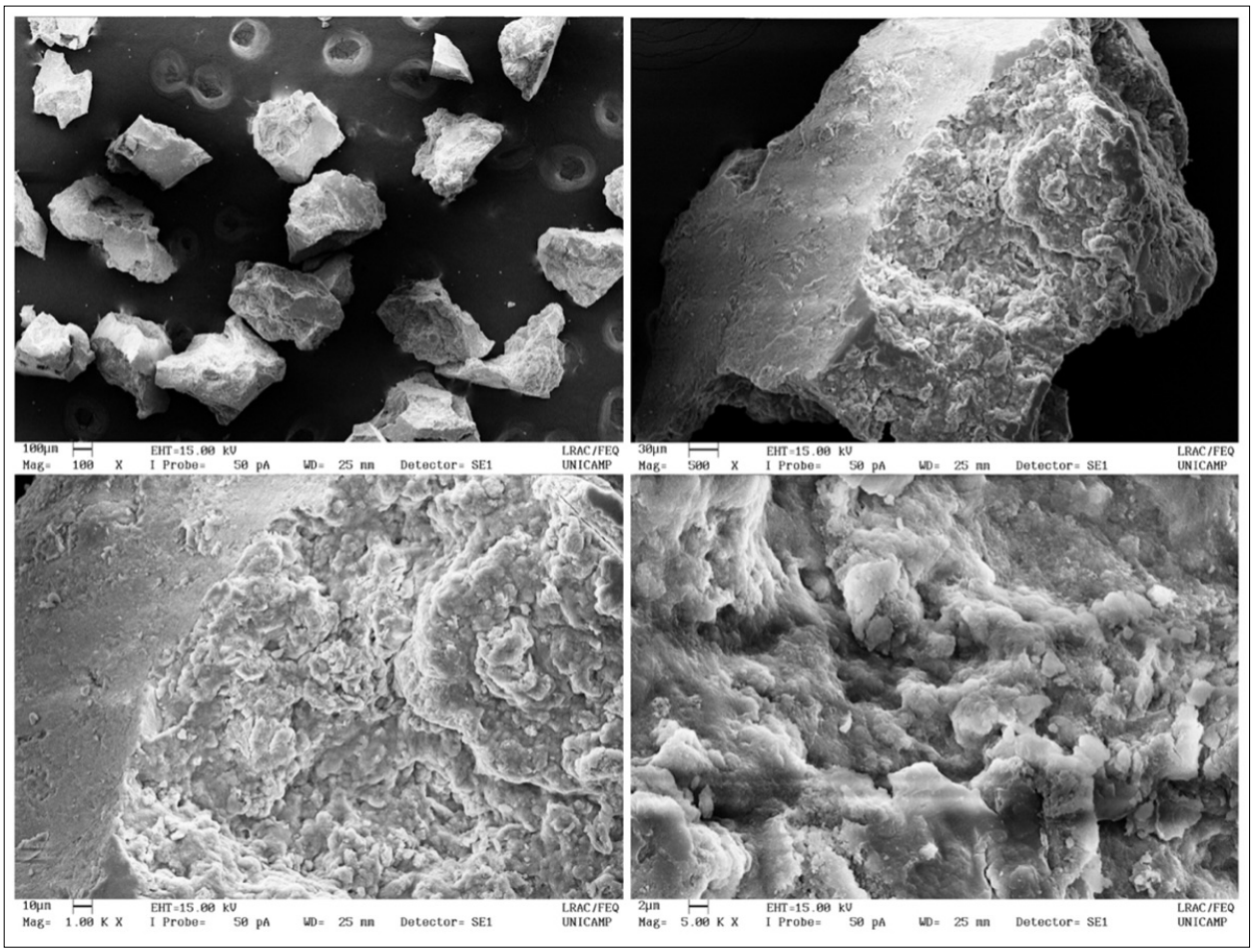

Fig. 3: SEM images of irregularly shaped CXHAG granules with surface microroughness.

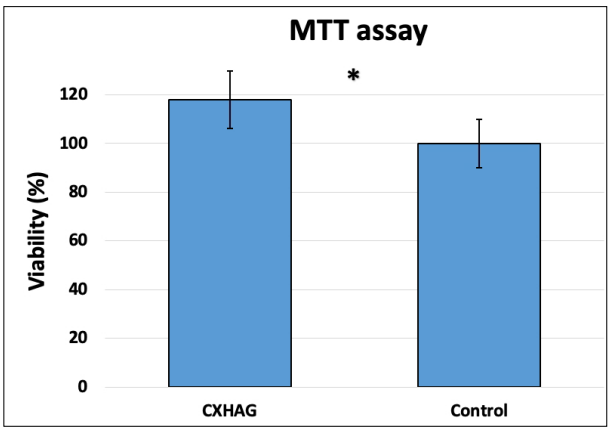

*Fig. 4: Indirect cytotoxicity WST-1 of Hydroxyapatite-Chitosan-Xanthan-Graphene Oxide Composite after 24-hour exposure to hDPSCs. *p<0.05. CXHAG = chitosan-xanthan-hydroxyapatite-graphene oxide.*

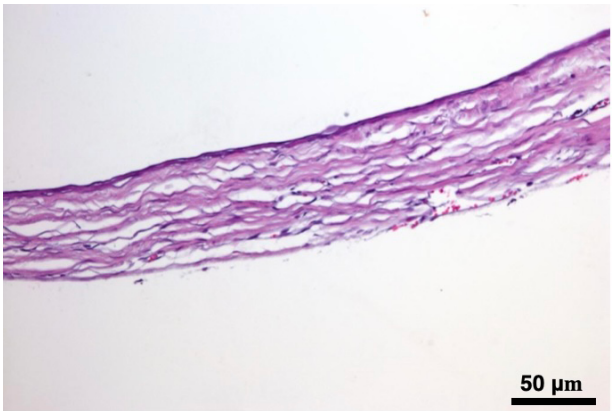

Fig. 5: Photomicrograph of histological section showing no bone formation at the center of the defect in the CG, after 42 days. (Scale bar = 50 µm).

In vivo assessment

Concerning the histologic findings, the CG showed the presence of loose immature connective tissue and no bone formation at the center of the defect (Fig. 5).

G1 presented a remnant of biomaterial particles at both timepoints, always surrounded by loose immature connective tissue. Multinucleated giant cells were observed around some biomaterial particles, especially at 14 days. There was a typical mononuclear inflammatory infiltrate at T1, but it

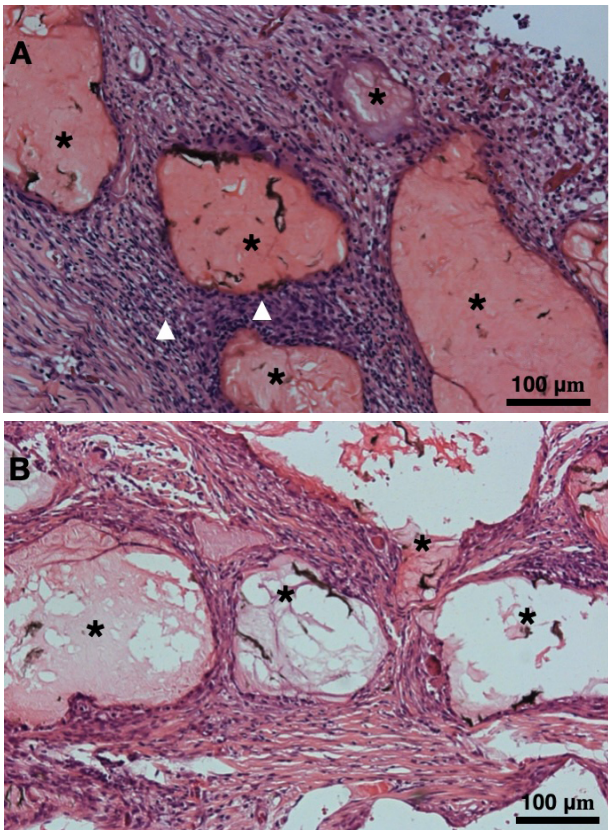

Fig. 6: Photomicrographs of histological sections showing no bone formation at the center of the defect in G1. Remnants of the biomaterial (*) and inflammatory infiltrate (arrowhead). A) 14 days and B) 42 days. (Scale bar = 100 µm).

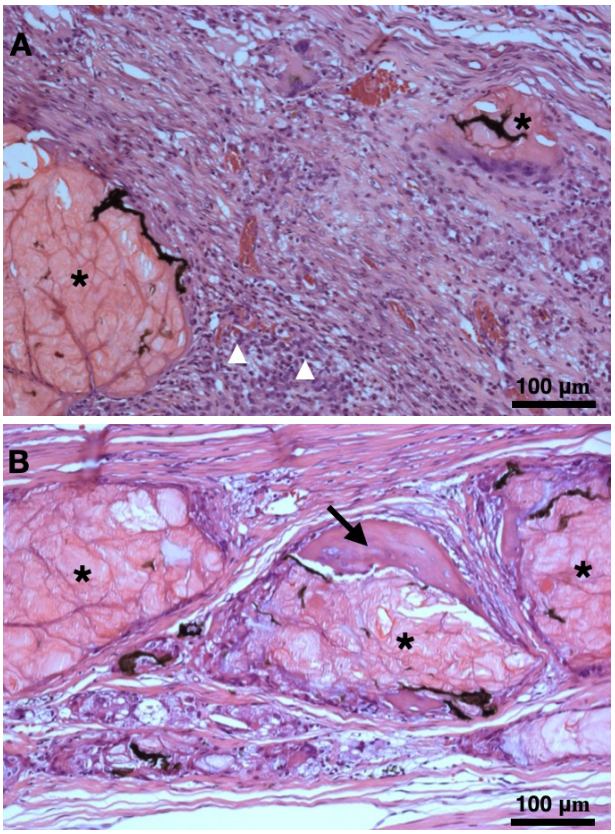

Fig. 7: Photomicrographs of histological sections showing bone formation (arrow) at the center of the defect in G2. Remnants of the biomaterial (*), inflammatory infiltrate (arrowhead), and newly formed bone (arrow). A) 14 days and B) 42 days. (Scale bar = 100 µm).

was attenuated at 42 days (T2) (Fig. 6).

G2 presented a remnant of biomaterial particles at both timepoints, but some bone formation was detected around the biomaterial particles only after 42 days. Of the total area evaluated, the median percentage of newly formed bone was 5.81% (1.95% minimum and 12.25% maximum). Multinucleated giant cells were observed around some biomaterial particles, especially at 14 days. Typically, a mononuclear inflammatory infiltrate was observed on T1. However, at 42 days (T2), there was a decrease in the inflammatory infiltrate (Fig. 7).

DISCUSSION

In the present work, we tested CXHAG granules covered by membranes in rat calvarial critical size defects. We found that bone formation in the center of the critical defect was stimulated only when the granules were supplemented by the conditioned medium from mesenchymal stem cells.

The present study replicated the production of CXHAG membranes previously described by our group[25]. Although the production protocol of these membranes aligns with our earlier publication, this study further investigates our new approach for producing CXHAG granules and their significance in addressing critical bone defect regeneration. The use of a bone substitute biomaterial filling defect and coverage with a membrane is the basis of the guided bone regeneration concept[26] and, therefore, this study adopted it by using CXHAG based biomaterials, either associated or not associated with the conditioned medium derived from mesenchymal stem cell. However, it is important to state that the membranes used in this study were not fixed and, therefore, some micromotion may have occurred, which might have impaired bone regeneration.

The diffraction patterns and FTIR spectra confirmed the chemical composition of the CXHAG granulated biomaterial. Moreover, the sizes of the CXHAG granules produced were predominantly within the 300 to 400 micrometer range, which is

consistent with the current trend in the literature that shows small size granules performing better than large ones[27-28]. A comprehensive examination under varied magnifications revealed distinctive surface topography at the micrometric scale. The irregularity in the granule shape was intended to potentially provide mechanical interlocking and to increase the specific surface area for enhanced cellular interactions. We hypothesized that the design would promote cell adhesion and proliferation. In this regard, this work provided evidence of a higher cellular response in terms of cell viability, which supports this hypothesis, since cell viability percentage was higher in CXHAG than in the control. In this regard, *in vitro* cell cytotoxicity assays utilizing WST play an indispensable role in predicting clinical toxicity. These colorimetric assays provide crucial preliminary data on the potential impacts of a biomaterial on cellular metabolic activity, effectively serving as a proxy for cell health and viability.

In this study, the results of WST-1 assay suggested that CXHAG granules exhibited high cell viability after 24 hours, with significant statistical difference ($p < 0.05$) in comparison to the control cell group. The presence of ions might have contributed to these findings. This result agrees with Souza et al.[25], who showed that hydroxyapatite-chitosan-xanthan-graphene oxide membrane had excellent properties for the cell viability evaluated by the MTT assay. The presence of multinucleated giant cells surrounding some biomaterial particles was expected, as it is a common finding when hydroxyapatite biomaterials are used, especially at early timepoints[29].

The present study provides evidence that the medium conditioned by stem cells obtained from dental pulp stimulated osteogenesis in the center of the defect after 42 days in this experimental model. These findings corroborate our previous study [16], which used the same medium but associated with a bovine hydroxyapatite bone graft, and was also designed with critical size defects and the same 2 timepoints as used in the present research. It is thus feasible to compare the two studies, enabling the understanding that both bone substitute biomaterials (i.e., hydroxyapatite, chitosan, xanthan and graphene oxide versus exclusive bovine hydroxyapatite) can be used as scaffolds for bone tissue engineering purposes. Both the present study and Buss et al.[16] showed no bone formation in the center of the critical sized bone defect with the use of grafts without addition of the conditioned medium, even after 42 days of healing in rat calvaria. These results highlight the inability of osteoconductive biomaterials (e.g., alloplastic or xenogenous bone grafts) to promote bone regeneration in critical areas without the use of tissue engineering/cell therapy approaches.

In the present study, after 14 days, no bone formation was observed in any group, showing that this timepoint is too early for bone regeneration to have occurred, probably due to the size of the critical defect, and consequently, the large distance between the bone walls. However, as bone formation in the center of the defect was only seen after 42 days in G2, and as the presence or absence of the conditioned medium was the only difference between G1 and G2, it can be stated that the medium was the only factor responsible for the difference in osteogenesis between groups. Recent studies have attributed such regenerative effects to the paracrine factors secreted by such cells[30]. Thus, the conditioned medium in which these cells are proliferated has extracellular vesicles and exosomes that might propagate the main regenerative and immunoregulatory characteristics[17,31-32]. The role of the osteogenic conditioned medium in bone formation in the center of the defect is explained by the release of bone regulatory proteins[33]. The presence of exosomes in the osteogenic conditioned culture medium can sensitize cells. However, it is important to note that the level and composition of extracellular vesicles and exosomes in the conditioned medium were not evaluated in this study.

The histological results in the CG (unfilled defect) after 42 days, showing no bone formation, make it clear that the defect used in the present study was a critical size bone defect. For critical size bone defects, the scientific literature shows that the use of tissue engineering concepts can promote earlier bone formation[12-13]. Critical size bone defects enable determination of whether the applied therapies are in fact promoting bone regeneration. It is worth mentioning that the literature considers a defect greater than 6 mm to be critical, since 6 mm is the threshold for spontaneous bone formation, which can occur after 8 weeks[34]. This fact, taken together with the histological results of the CG specimen, makes it clear that the 8mm defect used in the present study really represents a critical bone defect, and therefore

supports the statement that the only variable used in this study (i.e., the conditioned medium in from mesenchymal stem cells) was responsible for the bone formation in the center of the critical bone defect. In this regard, it is important to note that a higher level of bone formation might occur at longer times (e.g., 8 weeks). However, Song et al.[35] showed that there was not complete bone regeneration of 8 mm defects, even after 8 weeks of healing. Moreover, the use of the critical size model healing evaluated at two timepoints enables the evolution of inflammatory infiltrate to be verified over time. In the present study, as in our previous publication[16] in which this same model with xenografts was used, the inflammatory infiltrate decreased between 14 days and 42 days. The trend to have an overrepresentation of the immune/inflammatory processes, with an upregulation of genes associated with leucocyte and T-cell activation at the early healing stages in critical size defects, has been demonstrated by other studies[36-37].

It is important to emphasize that the results of this study cannot be immediately extrapolated to clinical practice, since they derive from an animal model. Therefore, controlled randomized clinical trials are suggested to confirm the regenerating potential of the conditioned medium of mesenchymal stem cells when associated with different scaffolds. Further in vitro studies should be performed to evaluate the effect of the proposed granules on mineralized extracellular matrix as well as on the simulated body fluid[38], and in vivo studies to evaluate the degradation and porosity after bed implantation.

CONCLUSIONS

The biomaterial composed of hydroxyapatite, chitosan, xanthan, and graphene oxide presented irregular granules and showed an improvement in stem cell viability. The conditioned culture medium associated with this biomaterial was able to promote some bone regeneration in the center of critical bone defects in rat calvaria, in contrast to the biomaterial alone.

CONFLICT OF INTEREST

The authors declare no conflicts of interest concerning the publication of this article

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