FULL ARTICLE

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In vitro evaluation of hydroxyapatite, chitosan, and carbon nanotube composite biomaterial to support bone healing

Avaliação in vitro do biomaterial compósito à base de hidroxiapatita, quitosana e nanotubo de carbono como adjuvante no reparo ósseo

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ABSTRACT

Hydroxyapatite, chitosan, and carbon nanotube composite biomaterial were developed to improve bone healing. Previous studies suggested that a combination of biomaterials and mesenchymal stem cells (MSCs) can potentially help promote bone regeneration. In the present study, we first developed hydroxyapatite, chitosan, and carbon nanotube composite biomaterial. Then, the effect of different concentrations of the extract on the viability of Vero cells (ATCC CCL-81) and MSCs obtained from sheep bone marrow using methylthiazol tetrazolium (MTT) and propidium iodide (PI) assays were evaluated. The biomaterial group demonstrated an absence of cytotoxicity, similar to the control group. Samples with 50% and 10% biomaterial extract concentrations showed higher cell viability compared to samples from the control group (MTT assay). These results suggest that the presence of this composite biomaterial can be used with MSCs. This study also concluded that hydroxyapatite, chitosan, and carbon nanotube composite biomaterial were not cytotoxic. Therefore, these could be used for performing *in vivo* tests.

Keywords: Bone regeneration. Propidium iodide assay. Methylthiazol tetrazolium assay. Mesenchymal stem cells. Cytotoxicity.

RESUMO

O compósito à base de hidroxiapatita, quitosana e nanotubo de carbono foi desenvolvido com o intuito de auxiliar na consolidação óssea. Estudos anteriores sugerem que a combinação de substitutos ósseos e células-tronco mesenquimais (CTM) podem auxiliar a potencializar e promover a regeneração óssea. No presente estudo, o biomaterial foi desenvolvido e a viabilidade e a citotoxicidade de células Vero (ATCC CCL-81) e CTM obtidas de medula óssea provenientes de ovinos utilizando ensaios metil-tiazol-tetrazólio, MTT e iodeto de propídeo (PI) foram avaliadas em diferentes concentrações de extrato desse compósito. O compósito demonstrou ausência de citotoxicidade com comportamento semelhante ao grupo controle. Amostras com 50% e 10% de concentração de extrato do compósito mostraram resultados maiores comparados ao grupo controle (ensaio MTT). Esses resultados também sugerem que a presença do biomaterial pode ser utilizada em associação a CTM. Assim, esse estudo conclui que o compósito apresentado de hidroxiapatita, quitosana e nanotubo de cabono não foi considerado citotóxico e pode ser utilizado em teste *in vivo.*

Palavras-chave: Regeneração óssea. Iodeto de propídeo. Metil-tiazol-tetrazólio. Célula-tronco mesenquimal. Citotoxicidade.

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Introduction

Numerous synthetic and biologically derived materials have been evaluated for use in the preservation or augmentation of bone defects (Emara et al., 2013). Several studies investigated biomaterials for use in bone tissue engineering by employing different methodologies (Marcondes et al., 2016; Milori et al., 2013; Nóbrega, 2014; Paretsis et al., 2017).

Hydroxyapatite (HA) is a well-known biomaterial component that is widely used because of its similarity to bone tissue, biocompatibility, and osteoconductivity (Azevedo et al., 2013; Dantas et al., 2011; Ferreira et al., 2017; Notodihardjo et al., 2012; Reis et al., 2010). Chitosan is an easily processed biopolymer, known for its excellent biocompatibility, as well as antioxidant and antimicrobial properties (Spin-Neto et al., 2012; Tavakol et al., 2013; Tavaria et al., 2013; Tsuchiya et al., 2014). Carbon nanotubes have been used with both of these biomaterials to improve mechanical resistance and assist with bone osteoconduction (Barrientos-Durán et al., 2014; Shin et al., 2011).

A composite of HA, carbon nanotubes and chitosan has been previously prepared, although different raw material sources and procedures were used. Since these biomaterials have a uniform distribution and biocompatibility, they are considered potentially promising for applications in bone tissue engineering (Chen et al., 2013; Venkatesan et al., 2011).

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into different types of tissues and can be used to support tissue regeneration. MSCs are precursors of bone, cartilage, muscle, and fat cell lineages (Freitas et al., 2017; Portinho et al., 2006). The therapeutic use of MSCs is still highly promising for bone regeneration. More studies have been conducted on the utilization of MSCs and bone graft substitutes in combination, with an effort to engineer an optimal microenvironment for the regeneration and repair of damaged bone tissue (Lee et al., 2019).

In vitro experiments allow us to simulate and predict biological reactions to implanted materials. Therefore, they should be performed first before *in vivo* biocompatibility tests. These *in vitro* tests enable the assessment of biomaterial cytotoxicity and reduce the need for animal models (Fidalgo et al., 2009; Masson et al., 2014; Schönberger et al., 2007). Biomaterials interacting with host cells and/or tissues can cause numerous biological responses, such as altered cell morphology, metabolic activity, and behavior, affecting the potential clinical applications of these biomaterials (Masson et al., 2014).

In the present study, we evaluated the effect of HA, chitosan, and carbon nanotube composite scaffolds on the cytotoxicity and viability of Vero cells (ATCC CCL-81) and MSCs obtained from sheep bone marrow *in vitro* using methylthiazol tetrazolium (MTT) and propidium iodide (PI) assays.

This preclinical test aimed to determine if this biomaterial has any grade of cytotoxicity. We hypothesized that there is no high level of toxicity in different concentrations of the composite biomaterial for Vero cells and MSCs.

Materials and methods

The experimental design was proposed to describe the production and characterization of a biomaterial composite scaffold. MSCs were collected and differentiated to confirm the quality of the biomaterial used as a scaffold. MSC cultures with the biomaterials were performed to evaluate the biocompatibility of the biomaterial concerning the growth of MSCs. Cell viability was assessed by MTT and PI assays using the Vero cell line (ATCC CCL-81) and MSCs derived from sheep bone marrow, which was performed in triplicate. Vero cells were chosen as the first approach based on the study by Amaral et al. (2020) because they are easy to obtain and manipulate.

Preparation of biomaterial composite scaffold

Hydroxyapatite was synthesized from calcium nitrate and ammonium phosphate as previously described (Jarcho et al., 1976). X-ray diffraction, performed using an X-ray diffractometer (Bruker D8 Advance Instrument, Karlsruhe, Germany), showed that the diffraction peaks matched those of the HA standard [HA, JCPDS 9-0432]. Energy-

dispersive X-ray analysis, performed using an EDX LINK ANALYTICAL system (Isis System Series 200, Cambridge, England) coupled to an electronic microscope LEO 440 (LEO Electron Microscopy Ltd, Cambridge, England), Oxford detector (Oxford Instruments Inc., Cambridge, England), demonstrated a calcium/phosphorus (Ca/P) ratio of 1.89, consistent with a calcium-rich HA (Ramesh et al., 2007). Chitosan was obtained by deproteinization and demineralization of squid gladius (*Doryteuthis spp.*). The molecular weight (MW) and degree of deacetylation (DD) were determined by viscosimetric and ^{1}H NMR spectroscopy, respectively. The DD value was 93.5% (Horn et al., 2009) and the MW was 2.83 ± 0.6×10^{5} Da. Carbon nanotubes (CNTs) were multi-walled, had a 9.5 nm diameter and 1.5 μm length, and were functionalized with carboxylic acid (>8%) (Aldrich, Saint Louis, USA). To prepare the scaffold, CNTs were suspended in 1% acetic acid. Chitosan was then slowly added and stirred at 2000 rpm for 24 h to achieve complete dissolution. The same procedure was performed in adding HA. The final ratio of the biomaterials was 1:20:180 (carbon nanotubes:chitosan:HA).

To prepare the scaffolds, 100 mg and 500 mg of the solution were placed into Teflon® molds with dimensions of 70 mm diameter × 2 mm height and 100 mm diameter × 2 mm height, respectively, frozen in liquid nitrogen, and lyophilized in an Edwards model Freeze Dryer Modulyo (Edwards High Vacuum International, West Sussex, UK). Matrices were neutralized in a 0.1 mol/L $NaHCO_3$ solution followed by multiple washes with deionized water and subsequent freezing and lyophilization (Figure 1).

Characterization of scaffolds

Morphological evaluation of the scaffold was performed using scanning electron microscopy (SEM). The sample was mounted on aluminum stubs with carbon tape, sputter-coated with Au/Pd using a Coating System BAL-TEC MED 020 (BAL-TEC, Liechtenstein) under 2.00 × 10^{-2} mbar pressure, 60 mA current, and 0.60 nm s^{-1} deposition rate. ZEISS LEO 440 (Cambridge, England) equipment and OXFORD (model 7060) detector, operated at a voltage of 20 keV, were used. Images were acquired using a quadrant backscattered electron detector (QBSD) type 400 at 2.82 A current and with an I 1500 nA probe.

Isolation of mesenchymal stem cells from sheep bone marrow

Aspiration of the iliac crest bone marrow obtained from a two-month-old Dorper sheep was performed to isolate MSCs.

Bone marrow was collected in 20 mL vials coated with sodium heparin. Immediately, the blood was diluted with phosphate-buffered saline (PBS) and layered over in Ficoll Histopaque®-1077 (1.077 g/mL) in a 1:1 ratio. The mononuclear fraction was harvested after density gradient centrifugation for 30 min at 1400 × g. The mononuclear cells were rinsed in the same volume of PBS and then centrifuged for 10 min at 2000 × g. Subsequently, the samples were rinsed again using the same conditions.

The cell pellets were suspended and then plated in a basal medium consisting of 5 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% pyruvic acid, 1% L-glutamine, 1% insulin, and 0.5% B-amphotericin (LGC, Biotechnology, Brazil). The cells were cultured in control media until 80% confluence at 37 °C and 5% CO_2 in a humidified incubator. Next, the cells were trypsinized and frozen at -80 °C for 24 h and then at -196 °C in liquid nitrogen.

To verify the multipotentiality of MSCs, the cells were differentiated into osteoblasts, adipocytes, and chondrocytes according to standard protocols (Burk et al., 2017; Fülber et al., 2016).

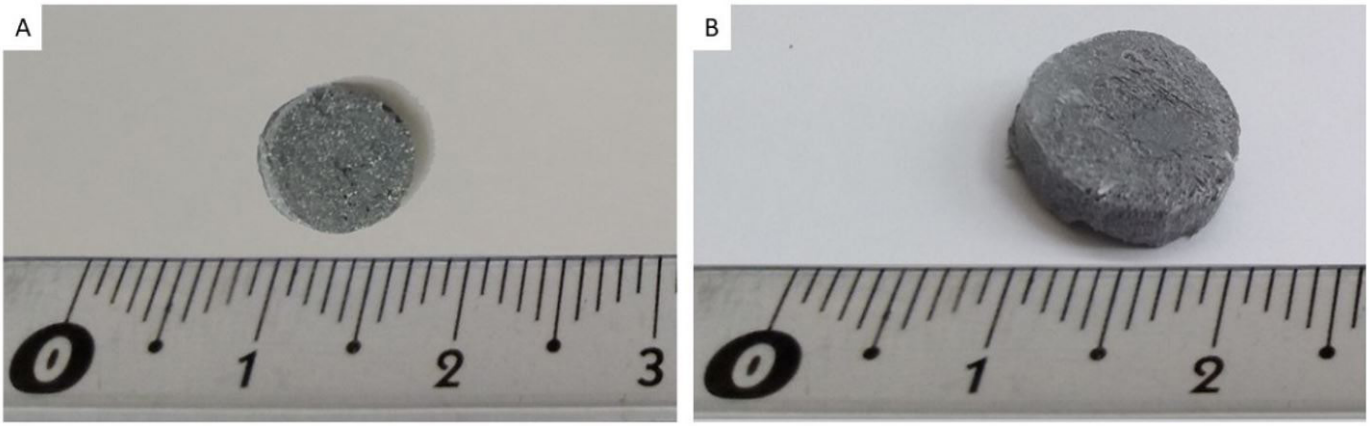

Figure 1 - Hydroxyapatite, chitosan, and carbon nanotube disk biomaterial composite (70 mm diameter x 2 mm height) (A) and 100 mm diameter x 2 mm height (B).

Mesenchymal cell culture with biomaterials

The analysis of the biocompatibility of the biomaterials, concerning the growth of MSCs, was performed using two protocols to confirm if the biomaterial had any inert or harmful characteristics after their interaction. The MSCs were placed directly on top of the biomaterials in culture flasks (2D). Then, the MSCs were cultured until 70-80% confluence (about eight days). The second protocol was carried out using MSCs already attached to the culture flasks. For this, the biomaterial was added to the flask and allowed to maintain contact with the cells in culture until a confluence of 70-80% was attained.

Effect of the biomaterial on cell viability (MTT assay)

The viability of cultured cells was determined using the colorimetric MTT assay according to the manufacturer's protocol (Amaral et al., 2020). Two types of cells were used for this assay, the Vero cell line (ATCC CCL-81) and MSCs.

The cells were seeded onto 96-well plates at a density of 1×10^5 cells/mL and incubated under standard culture conditions. Extracts from the biomaterials were obtained according to ISO 10993-12 standards. Extracts from the composite biomaterial were prepared by incubating the pre-sterilized biomaterial (7 mm × 1 mm size) in 2 mL of the DMEM media for 24 h at 37 °C. The extract solution was prepared at different concentrations in the media previously described (Duarte, 2015).

Cells were incubated with different concentrations of the extract (100%, 90%, 75%, 60%, 50%, 25%, and 10%) for 48 h. Latex at concentrations of 100% and 50% were subjected to the same conditions and used as a positive control. Undiluted culture media in DMEM was used as a negative control. At the end of the incubation period, the culture medium was replaced with fresh media containing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) solution.

The media was removed after 2 h and the reaction was terminated by the addition of 100 µL dimethyl sulfoxide (DMSO) to each well as a solubilization buffer to dissolve the formazan crystals. The lysate absorbance was then measured using a microplate reader (ELISA BIO-RAD) at 500 nm.

Propidium iodide (PI assay)

The Vero cell line and the MSCs were cultured and incubated as previously described, and then stained with PI. After 15 min of incubation at 37° C, the cells were immediately observed using an inverted fluorescence microscope (Carl Zeiss, Inc, Germany).

Statistical analysis

The results were expressed as the triplicate means ± standard deviation and analyzed using GraphPad Prism software Version 6. Statistical analysis was performed using one-way ANOVA, and differences were considered significant at $^*p < 0.05$.

Results

Characterization of the scaffold

SEM images (Figure 2) of the scaffold surface show a compact composite with distributed materials.

Isolation of mesenchymal stem cells

The isolated MSCs from bone marrow cultures demonstrated growth, proliferation, and adhesion in seven

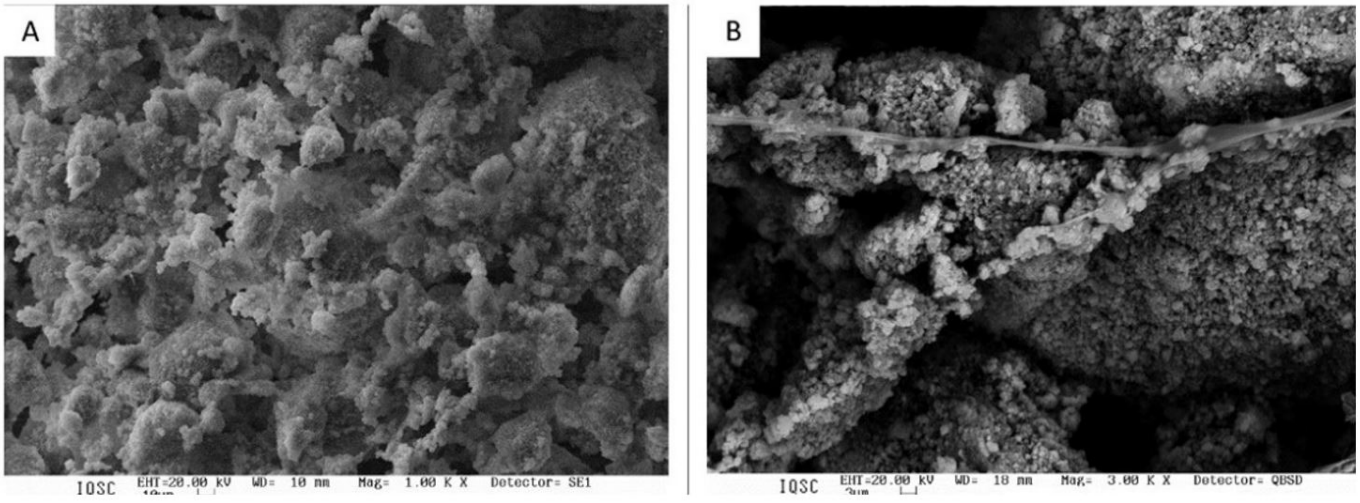

Figure 2 - SEM photomicrograph of the scaffold. (A) 1000× magnification, scattering detector, and (B) 3000× magnification, quadrant backscattered electron detector.

days. MSCs had a fibroblastic morphology and grew as a monolayer. After 15 days, the cell cultures reached ~70% confluence (Figure 3A).

Mesenchymal stem cell culture with biomaterials

Mesenchymal stem cells cultured with biomaterials since the beginning of the experiments were able to adhere and grow according to the first protocol. Adhesion and growth on the MSC culture with biomaterials were also observed (Figure 3B).

For the second protocol, where the biomaterial was introduced after the MSCs were confluent, similar results were observed. No cell death was detected.

MTT assay

Figure 4A presents the MTT results for Vero cells in P3. There was no difference in cell viability between the control group (blank) and the different biomaterial extract concentrations. The groups with 50% and 10% concentrations showed higher cell viability than the control group. None

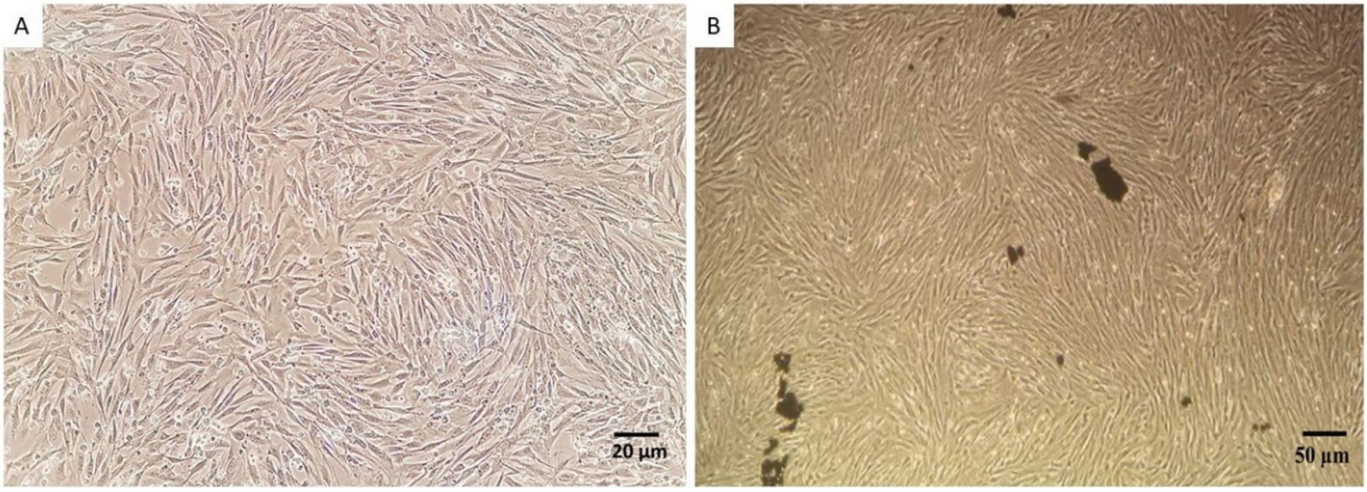

Figure 3 - Representative images of MSCs obtained from sheep bone marrow during cell culture (P3) showing ≥ 100% confluence in the region. MSC cultures observed using an inverted microscope at 10× magnification (A); MSCs on biomaterials as observed using an inverted microscope at 4× magnification (first protocol) (B).

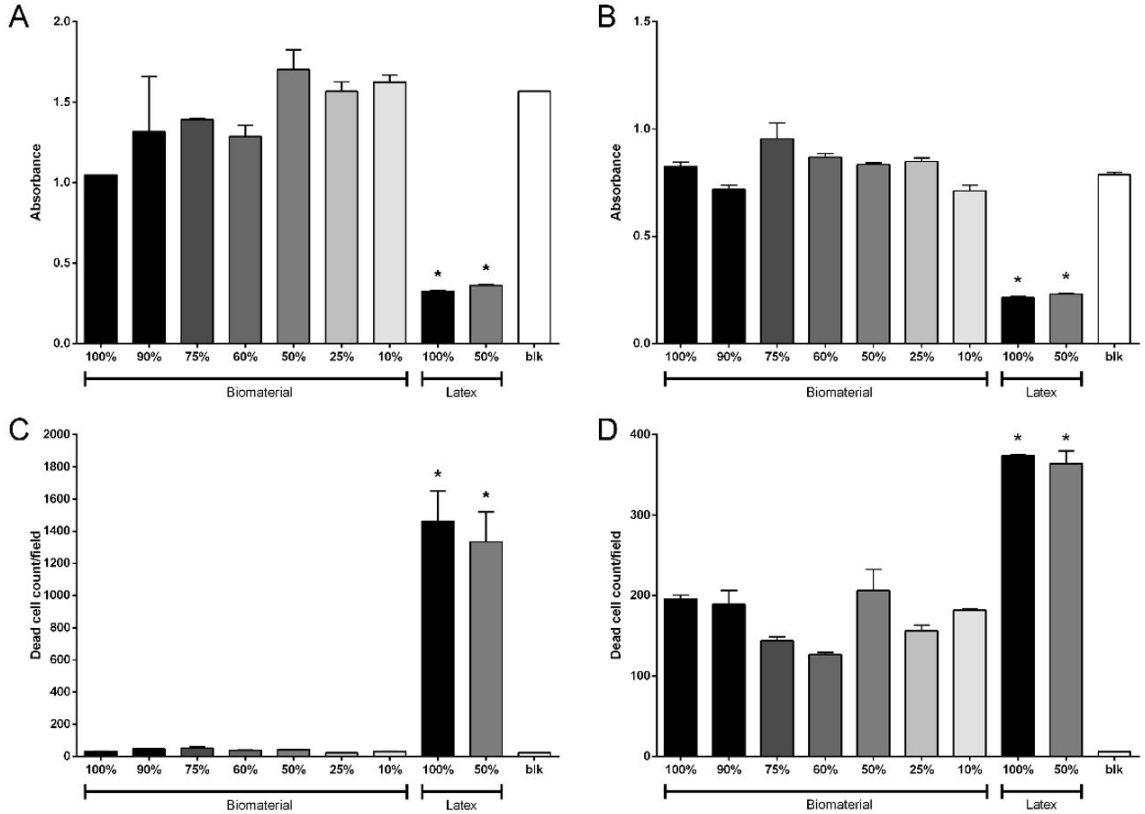

Figure 4 - MTT absorbance with Vero cells (A); MTT absorbance with MSCs (B); Dead cell count with Vero (PI assay) (C); Dead cell count with MSCs (PI assay) (D).

of the groups presented absorbances similar to the positive control (latex).

Figure 4B shows the MTT assay results for the MSCs at P3. There was an increase in viable cells in the presence of biomaterial scaffolds at different concentrations, with the absorbances in groups with 100%, 75%, and 25% concentrations higher than the control group.

PI assay

As expected, the PI assay in Vero cells demonstrated the presence of dead cells in the positive control group. The samples from the negative control group (blank), as well as the samples containing various concentrations of the biomaterial extract, showed a similar number of dead cells (Figure 4C).

Meanwhile, the PI assay performed with the MSCs showed that the number of dead cells in the biomaterial-containing samples was higher than that in the negative control group (blank). However, the number of dead cells in the biomaterial-containing samples was lower than that in the positive control group (latex) (Figure 4D).

Discussion

Although materials such as HA, chitosan, and carbon nanotubes are well known, only a few studies have examined the composite biomaterial containing all three materials (Chen et al., 2013; Türk et al., 2018). To develop this type of biomaterial, natural raw materials were used to extract chitosan, and HA was synthesized in the laboratory. The only commercial material was carbon nanotubes, which were included to improve material resistance.

In this study, HA, chitosan, and carbon nanotube composite biomaterial were evaluated *in vitro* for their effects on cell viability and cytotoxicity. The MTT and PI assays demonstrated the absence of cytotoxic effects in all tested cells.

The MTT assay measures the reduction of the tetrazolium component MTT by viable cells (Patel et al., 2009; Türk et al., 2018). Therefore, the level of reduction reflects the level of cell metabolism, quantifying mitochondrial activity (Gupta et al., 2017). The MTT assay demonstrated that cells cultured with different concentrations of the biomaterial extract had similar levels of metabolic activity compared to the control.

Our results corroborate the findings of Masson et al. (2014). Their group used the MTT assay to test the viability of NCTC Clone 929 (ATCC/CCL-1) and Vero (ATCC/CCL-81) cells on different biomaterials (samples of Bioglass® 45S5 and glass-ceramics from the $3CaO.P_2O_5$-SiO_2-MgO system). The authors considered the biomaterials to be non-cytotoxic and emphasized the good quantitative characteristics of this assay. Previously, similar MTT results were obtained for other bone regeneration biomaterials, and no distinction was observed between the negative control and the biomaterial extract samples (Ahmad et al., 2017; Sun et al., 2018).

In the present study, the MTT assays showed that Vero cells at 50% and 10% concentrations of biomaterial extract had higher absorbance compared to those of the control group. MSCs also had higher absorbance levels than the control group at 100% and 75% – 25% concentrations of the biomaterial extract. These results suggest that biomaterial presence can be considered a favorable environment for cells. Similar conclusions were also reached in a cytotoxicity study performed with HuGu cells (human gum fibroblasts) by MTT assay for 72 h. These results showed higher cell proliferation and absence of cytotoxicity (Abd-Khorsand et al., 2017).

In the cytotoxicity assay, PI can only interact with nuclear DNA from cells with disrupted cytoplasmic and nuclear membranes, staining the nuclei red (Pan et al., 2012). The cytotoxicity of the different concentrations of the biomaterial extract in cultured MSCs was lower than that of the positive control group. It could be that MSCs were more sensitive to the presence of the biomaterial extract. However, this biomaterial cannot be considered significantly cytotoxic because the cell death in the positive control (latex) was significantly higher than the different concentrations of the biomaterial extract. For these reasons, several cytotoxicity methods are recommended to be used to provide complete information about the material under investigation (Jorge et al., 2004).

In the present study, Vero cells demonstrated similar results to MSCs as determined by MTT and PI assays. Nevertheless, other studies evaluated the effects of different bone-grafting materials on MSC culture using MTT and PI assays to conclude that their respective biomaterials presented a favorable microenvironment that improved adhesion, proliferation, and differentiation of the studied cells (Gupta et al., 2017; Pan et al., 2012).

It was not possible to use osteogenic cells in this study and there was no positive control in the MSC cultures with unknown objects. This could be considered a study limitation, although the composite biomaterial demonstrated in this preclinical study showed pertinent results.

In vitro and *in vivo* studies are excellent tools to evaluate the potential of the combination of the biomaterial and MSCs to improve biomaterial osseointegration and increase

tissue neoformation, with a quantity and quality equal to or better than the control group (Freitas et al., 2017; Portinho et al., 2006).

Comparing these results with those of previous studies (Ahmad et al., 2017; Masson et al., 2014; Sun et al., 2018), HA, chitosan, and carbon nanotube composite biomaterial was not considered cytotoxic. This composite biomaterial appears to provide a favorable environment for use in association with sheep-derived MSCs to assist tissue regeneration. However, more *in vitro* and *in vivo* studies are needed to confirm the use of this biomaterial as a bone substitute.

Conflict of Interest

The authors state that they have no conflicts of interest to declare.

Ethics Statement

Not applicable.

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References

Abd-Khorsand S, Saber-Samandari S, Saber-Samandari S. Development of nanocomposite scaffolds based on TiO 2 doped in grafted chitosan/hydroxyapatite by freeze drying method and evaluation of biocompatibility. Int J Biol Macromol. 2017;101:51-8. http://dx.doi.org/10.1016/j.ijbiomac.2017.03.067. PMid:28315764.

Ahmad N, Bharatham HB, Hamid ZA, Zulkipli NZ. Cytotoxicity and oxidative stress evaluation of alginate/cockle shell powder Nanobiocomposite bone scaffold on osteoblast. J Sains Kesihat Malaysia. 2017;15(1):97-103. http://dx.doi.org/10.17576/JSKM-2017-1501-12.

Amaral MB, Viana RB, Viana KB, Diagone CA, Denis AB, De Guzzi Plepis AM. In vitro and in vivo response of composites based on chitosan, hydroxyapatite and collagen. Acta Sci Technol. 2020;42(1):1-13.

Azevedo AS, de Sá MJC, Fook MVL, Neto PIN, de Souza OB, Azevedo SS. Hidroxiapatita e quitosana isoladas e associadas à medula óssea no reparo do tecido ósseo em coelhos. Estudo histológico e morfométrico. Ciência Rural St Maria. 2013;43(7):1265-70. http://dx.doi.org/10.1590/S0103-84782013000700019.

Barrientos-Durán A, Nieden NI, Malinin TI. Rodríguez- JC. Carboxyl-modified single-wall carbon nanotubes improve bone tissue formation in vitro and repair in an in vivo rat model. Int J Nanomedicine. 2014;4:4277-91. http://dx.doi.org/10.2147/IJN.S62538. PMid:25246785.

Burk J, Glauche SM, Brehm W, Crovace A, Francioso E, Hillmann A, Schubert S, Lacitignola L. Characterisation and intracellular labelling of mesenchymal stromal cells derived from synovial fluid of horses and sheep. Vet J. 2017;222:1-8. http://dx.doi.org/10.1016/j.tvjl.2017.02.006. PMid:28410670.

Chen L, Hu J, Shen X, Tong H. Synthesis and characterization of chitosan-multiwalled carbon nanotubes/hydroxyapatite nanocomposites for bone tissue engineering. J Mater Sci Mater Med. 2013;24(8):1843-51. http://dx.doi.org/10.1007/s10856-013-4954-x. PMid:23712535.

Dantas TS, Lelis ÉR, Naves LZ, Fernandes-neto AJ, de Magalhães D. Materiais de Enxerto Ósseo e suas Aplicações na Odontologia Bone Graft Materials and their Application in Dentistry. UNOPAR Cient Ciênc Biol Saúde. 2011;13(2):131-6.

Duarte CRA. Avaliação da citotoxicidade in vitro de composições de fosfato de cálcio para uso em reparação óssea [tese]. Lages: Universidade do Estado de Santa Catarina; 2015.

Emara SA, Gadallah SM, Sharshar A. Evaluation of coral wedge and composite as bone graft substitute to induce new bone dormation in a dog tibial defect. J Am Sci. 2013;9(7):526-37.

Ferreira WS, Costa CMR, Ferreira SRB, Nunes SF. Propriedades estruturais e eletrônicas da hidroxiapatita a partir de cálculos de primeiros princípios. Engevista. 2017;19(1):194-201. http://dx.doi.org/10.22409/engevista.v19i1.820.

Fidalgo TK S, Barcelos R, Petrópolis DB, Azevedo BR, Primo LG. Silva Filho FC e. Citotoxidade de diferentes concentrações de hipoclorito de sódio sobre osteoblastos humanos Citotoxicity of different amounts of sodium hypochlorite on human cultured osteoblasts. Biologia (Bratisl). 2009;57(3):317-21.

Freitas GP, Lopes HB, Almeida ALG, Abuna RPF, Gimenes R, Souza LEB, Covas DT, Beloti MM, Rosa AL. Potential of Osteoblastic Cells Derived from Bone Marrow and Adipose Tissue Associated with a Polymer/Ceramic Composite to

Repair Bone Tissue. Calcif Tissue Int. 2017;101(3):312-20. http://dx.doi.org/10.1007/s00223-017-0282-3. PMid:28451713.

Fülber J, Maria DA, Silva LCLC, Massoco CO, Agreste F, Baccarin RYA. Comparative study of equine mesenchymal stem cells from healthy and injured synovial tissues: an in vitro assessment. Stem Cell Res Ther. 2016;7(1):35. http://dx.doi.org/10.1186/s13287-016-0294-3. PMid:26944403.

Gupta SK, Kumar R, Mishra NC. Influence of quercetin and nanohydroxyapatite modifications of decellularized goat-lung scaffold for bone regeneration. Mater Sci Eng C. 2017;71:919-28. http://dx.doi.org/10.1016/j.msec.2016.10.085. PMid:27987789.

Horn MM, Martins VCA, Plepis AMG. Interaction of anionic collagen with chitosan: effect on thermal and morphological characteristics. Carbohydr Polym. 2009;77(2):239-43. http://dx.doi.org/10.1016/j.carbpol.2008.12.039.

Jarcho M, Bolen H, Thomas MB, Bobick J, Kay JF, Doremus RH. Hydroxylapatite Synthesis and Characterization in Sense Polycristalline Forms. J Mater Sci. 1976;11(11):2027-35. http://dx.doi.org/10.1007/PL00020328.

Jorge JH, Giampaoli ET, Pavarina AC. Citotoxicidade dos Materiais Dentários. Rev Odontol UNESP. 2004;33(2):65-8.

Lee CC, Hirasawa N, Garcia KG, Ramanathan D, Kim KD. Stem and progenitor cell microenvironment for bone regeneration and repair. Regen Med. 2019;14(7):693-702. http://dx.doi.org/10.2217/rme-2018-0044. PMid:31393221.

Marcondes GM, Nobrega FS, Corrêa L, Arana-chavez VE, Plepis AMG, Martins VCA, Zoppa ALV. Avaliação da interação biológica entre compósito de quitosana, colágeno e hidroxiapatita e tecido ósseo ovino. Arq Bras Med Vet Zootec. 2016;68(6):1531-8. http://dx.doi.org/10.1590/1678-4162-8824.

Masson AO, Nascimento MHM, Lombello CB. Análise comparativa de diferentes métodos de citotoxicidade in vitro. In: Proceedings of the 24º Congresso Brasileiro de Engenharia Biomédica – CBEB; 2014 Out 13-17; Uberlândia; 2014. p. 2484–7.

Milori FP, Quitzan J, de Souza RS, Cirio SM, Dornbusch PT, do Prado AMRB. Placas ósseas confeccionadas a partir de diáfise cortical equina na osteossíntese femoral em coelhos. Pesq Vet Bras. 2013;33(10):1201-7. http://dx.doi.org/10.1590/S0100-736X2013001000005.

Nóbrega FS. Avaliação da interação biológica entre polímero de poliuretana de mamona acrescido de carbonato de cálcio e tecido ósseo de equinos [tese]. São Paulo: Faculdade de Medicina Veterinária e Zootecnica, Universidade de São Paulo; 2014. http://dx.doi.org/10.11606/T.10.2014.tde-12112014-093337.

Notodihardjo FZ, Kakudo N, Kushida S, Suzuki K, Kusumoto K. Bone regeneration with BMP-2 and hydroxyapatite in critical size calvarial defects in rats. J Craniomaxillofac Surg. 2012;40(3):287-91. http://dx.doi.org/10.1016/j.jcms.2011.04.008. PMid:21737289.

Pan L, Pei X, He R, Wan Q, Wang J. Multiwall carbon nanotubes/polycaprolactone composites for bone tissue engineering application. Colloids Surf B Biointerfaces. 2012;93:226-34. http://dx.doi.org/10.1016/j.colsurfb.2012.01.011. PMid:22305638.

Paretsis NF, Arana-Chavez VE, Correa L, Peplis AMG, Martins VCA, Cortopassi SRG, Zoppa ALV. Avaliação histológica e histomorfométrica da regeneração óssea a partir da utilização de biomateriais em tíbias de ovinos. Pesq Vet Bras. 2017;37(12):1537-44. http://dx.doi.org/10.1590/s0100-736x2017001200029.

Patel S, Gheewala N, Suthar A, Shah A. In-vitro cytotoxicity activity of solanum nigrum extract against Hela cell line and Vero cell line. Int J Pharm Pharm Sci. 2009;1(Suppl. 1):38-46.

Portinho CP, Collares MVM, Silva FLH, Nardi NB, Pinto RA, Siqueira E, Morellato G, Sumino K. Reconstrução de calota craniana com células - tronco mesenquimais indiferenciadas: estudo experimental. Rev Soc Bras Cir Plást. 2006;21(3):161-5.

Ramesh S, Tan CY, Hamdi M, Sopyan I, Teng WD. The influence of Ca/P ratio on the properties of hydroxyapatite bioceramics. In Proceedings SPIE 6423, International Conference on Smart Materials and Nanotechnology in Engineering; 2007 Nov 1. Harbin, China. p. 64233A.

Reis ECC, Borges APB, Fonseca CC, Martinez MMM, Eleotério RB, Morato GO, Oliveira PM. Biodegradation of a Hydroxyapatite-polyhydroxybutyrate Composite. Braz Arch Biol Technol. 2010;53(4):817-26. http://dx.doi.org/10.1590/S1516-89132010000400010.

Schönberger T, Hahn J, Kasten P, Südkamp NP, Fechner K, Pearce S, Niemeyer P. A novel software-based evaluation method for objective quantification of bone regeneration in experimental bone defects. Eur Cell Mater. 2007;14:92.

Shin US, Yoon I-K, Lee G-S, Jang W-C, Knowles JC, Kim H-W. Carbon nanotubes in nanocomposites and hybrids with hydroxyapatite for bone replacements. J Tissue Eng. 2011;2011:1-10. http://dx.doi.org/10.4061/2011/674287. PMid:21776341.

Spin-Neto R, Coletti FL, de Freitas RM, Pavone C, Campana-filho SP, Marcantonio RAC. Chitosan-based biomaterials used in critical-size bone defects : radiographic study in rat ' s calvaria. Rev Odontol UNESP. 2012;41(5):312-7. http://dx.doi.org/10.1590/S1807-25772012000500003.

Sun T, Wang M, Shao Y, Wang L, Zhu Y. The effect and osteoblast signaling response of trace silicon Doping Hydroxyapatite. Biol Trace Elem Res. 2018;181(1):82-94. http://dx.doi.org/10.1007/s12011-017-1031-1. PMid:28456913.

Tavakol S, Nikpour MR, Amani A, Soltani M, Rabiee SM, Rezayat SM, Chen P, Jahanshahi M. Bone regeneration based on nano-hydroxyapatite and hydroxyapatite/chitosan nanocomposites: an in vitro and in vivo comparative study. J Nanopart Res. 2013;15(1):1-16. http://dx.doi.org/10.1007/s11051-012-1373-8.

Tavaria FK, Costa EM, Pina-Vaz I, Carvalho MF, Pintado MM. A quitosana como biomaterial odontológico: estado da arte. Rev Bras Eng Bioméd. 2013;29(1):110-20. http://dx.doi.org/10.4322/rbeb.2013.002.

Tsuchiya N, Sato S, Kigami R, Kawano E, Takane M, Arai Y, Ito K, Ogiso B. Effect of a chitosan sponge impregnated with platelet-derived growth factor on bone augmentation beyond the skeletal envelope in rat calvaria. J Oral Sci. 2014;56(1):23-8. http://dx.doi.org/10.2334/josnusd.56.23. PMid:24739704.

Türk S, Altınsoy I, Çelebi Efe G, Ipek M, Özacar M, Bindal C. 3D porous collagen/functionalized multiwalled carbon nanotube/chitosan/hydroxyapatite composite scaffolds for bone tissue engineering. Mater Sci Eng C Mater Biol Appl. 2018;92:757-68. http://dx.doi.org/10.1016/j.msec.2018.07.020. PMid:30184804.

Venkatesan J, Qian Z, Ryu B, Ashok Kumar N, Kim S-K. Kumarc, Nanjundan Ashok Kima S-K. Preparation and characterization of carbon nanotube-grafted-chitosan – Natural hydroxyapatite composite for bone tissue engineering. Carbohydr Polym. 2011;83(2):569-77. http://dx.doi.org/10.1016/j.carbpol.2010.08.019.

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