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% of 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 à CTM. Assim, esse estudo conclui que o compósito apresentado de hidroxiapatita, quitosana e nanotubo de carbono 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.
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 osteoconductivity (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 1H 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 NaHCO₃ 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. ZEISSLEO 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% CO₂ 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).](image-url)
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).](image1)

![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).](image2)
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.P2O5-SiO2-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

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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.

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Conflict of Interest

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

Ethics Statement

Not applicable.

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