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Graphene oxide nanoribbons as nanomaterial for bone regeneration: Effects on cytotoxicity, gene expression and bactericidal effect

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ABSTRACT

Graphene oxide nanoribbons (O-GNR) surges as an interesting nanomaterial for biomedical applications due to feasibility to incorporate functional groups and possible bactericidal properties. Herein, high concentrations of O-GNR were biologically evaluated using human osteoblast cells and gram positive and negative bacteria. Briefly, our goal were to evaluate: (1) synthetic pathway, (2) characterization and (3) effects of O-GNR composition and structural factors as a new approach for biomedical applications. For this, O-GNR were produced combining chemical vapor deposition and oxygen plasma treatment of multiwalled carbon nanotubes. Then, we analyzed the bioactivity, cell viability, osteogenic differentiation, matrix mineralization, mRNA levels of the five genes related direct to bone repair and bactericidal effect of high concentrations of O-GNR (10 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1} and 300 μg mL^{-1}). Impressively, O-GNR showed no cytotoxic effects up to a concentration of 100 μg mL^{-1} and no gene expression alteration when used in its dose. We also observed that *S. aureus* and *E. coli* bacteria are susceptible to damage when incubated with 100 μg mL^{-1} of O-GNR, showing approximately 50% of bacterial death. We consider that O-GNR displays attractive properties when used at a suitable dose, displaying bactericidal effect and apparently lacking to cause damages in the bone repair process.

1. Introduction

Nanomaterials, including nanoparticles, are largely used as biomaterials due to their ability to mimic natural tissues and due to their recently identified bactericidal properties. Examples of such nanomaterials include silver [1,2], zinc oxide [3], magnesium oxide [4], titanium oxide [5,6], nanohydroxyapatite (nHAp) [7], carbon nanotubes (CNTs) [8], graphene oxide (GO) [9] and more recent GO nanoribbons (O-GNR).

CNTs and GO are especially unique materials of extensive interest in the nanomaterial world because of their biocompatible carbon-based chemistry and outstanding properties (such as high surface area, lightweight, exceptional mechanical elasticity, large carrier mobility, biocompatibility, ability for functionalization, and low thermal and electrical resistivity) [10]. In addition, GO and CNTs are currently being tested as engineered tissues [11,12], implants [12], diagnostic tools and chips, enhancing biological images [13], drug delivery carriers [14], and antibacterial materials [15].

Tour, and co-workers developed an oxidative method able to unzipped multiwalled carbon nanotubes to obtain GNR [16]. However, Chowdhury et al. was the first to analyzed the biological assays of O-GNR using different cell lines [17]. To date few papers related the relationship between cytotoxicity and expression of genes related to osteogenesis when applied high concentrations of CNTs, GO or O-GNR. In this way, Li et al. had evaluated different MWCNT-COOH concentrations using murine cementoblast. Then, 5 μg mL^{-1} of MWCNT-COOH promoted differentiation of alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin, type I collagen and *Runx2* gene expression without significant toxicity. However, when applied high concentrations (25 μg $mL^{-1}$of MWCNT-COOH) improved the cytotoxicity. The possible mechanism of the enhanced mineralization may be attributed to the up-regulation of key genes at different stages of cementum formation [18]. Meanwhile, Mahmood et al., reported that when MC3T3-E1 bone cells was exposure to carboxylated MWCNTs at different

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concentrations (1–10 μg mL^{-1}) resulted in enhancement of mineralization in a time-dependent manner. On the other hand, 30 μg mL^{-1} of carboxylated CNTs had presented toxicity effects for cells [19]. Other study showed that CNTs:HAp nanocomposites stimulated osteoblast cell proliferation. However, nanocomposites at higher doses (100 μg mL^{-1} and 500 μg mL^{-1}) showed cytotoxic effect [20].

To date is not totally comprehensive either functionalized carbon nanomaterials is toxic due to media agglomeration or improve the bone tissue regeneration due their physico-chemical properties. Also, it is not clear the cytotoxicity of their dose-dependency when incubated with cells. To pointing out these doubts, our group showed that MWCNT-GO presented no-toxicity using two different cell lines (NIH-3T3 and NG97), and had good efficiency for gene transfection at low concentrations [21]. Here, we presented an advanced from this recently published paper. We evaluated the cytotoxicity of different concentrations of O-GNR (10 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1} and 300 μg mL^{-1}) using MTT (3-(4,5-dimethylazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. We also analyzed the expression of genes involved in osteogenic by RT-qPCR and whether their concentrations could either present cytotoxic effects or improve the expression of gene related to osteogenesis. We also investigated the bactericidal effect of O-GNR using two very common gram-negative and gram-positive clinically-relevant bacteria. We noted, for the first time, that high doses of O-GNR O (up to 100 mg L^{-1}) showed no cytotoxic effect or a negative influence in the expression of some important genes of osteogenic pathway and a significant bactericidal effect.

2. Materials and methods

2.1. Synthesis and characterization of graphene oxide nanoribbons (O-GNR)

Briefly, we prepared multi-walled CNTs (MWCNT) using a mixture of camphor (85% wt) and ferrocene in a thermal chemical vapor deposition (CVD) furnace. The mixture was vaporized at 220 °C in an antechamber, and then, the vapor was carried by an argon gas flow at atmospheric pressure to the chamber of a CVD furnace set at 850 °C [8,21]. We removed the Fe catalytic particles using acid treatments. For this, we used ultrasound irradiation for 1 h in 10 M of HCl at 150 °C. We performed oxygen plasma treatment of the MWCNT to incorporate oxygen-containing groups and exfoliated them to produce O-GNR [22]. High resolution scanning electron microscopy (HR-SEM, FEI-TECNAI 200 kV $G2F_{20}$ Hillsboro Oregon, USA) and X-ray diffraction (XRD, using a diffractometer Panalytical X'Pert Pr, Lelyweg Almelo Netherlands) was used to characterize the O-GNR.

2.2. Bioactivity assay

We used 1.5× SBF (pH 7.4) to analyze the bioactivity of the O-GNR [23]. For this, we placed the O-GNR in a plastic container with SBF (1 mg mL^{-1}). We kept the samples immersed for two different time periods (14 and 21 days) at 36.5 °C in an orbital shaker incubator (Cientec© CT-712-R, Belo Horizonte MG, Brazil), with a constant agitation rate of 75 rpm. We monitored the pH of the solution up to 21 days using a pH meter (Metrohm Pensalab© 827). After that, we filtered the solution to recover the powder, and then we carefully washed it with deionized water at 80° for the removal of soluble inorganic ions and the salts. We evaluated the formed apatite's using Scanning Electron Microscopy (SEM, Zeiss - EVO MA10, Oberkochen, Germany). XRD evaluated the formed apatite's crystallographic properties. Fourier transform infrared spectroscopy (FTIR) using a Perkin Elmer Spotlight-400 (Waltham Massachusetts, USA) examined the presence of Ca and P. We used μEDX (model 1300 Shimadzu/Kyoto, Japan) to analyze the Ca and P ratio soaking the O-GNR in 1.5× SBF.

2.3. Cellular toxicity

MG-63 (ATCC® CRL-1427™) human osteoblast cell line was used in this study. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin was used. Cells were cultured at 37 °C in a 5%CO_2 humidified incubator. To assess cell viability, we used the exclusion test and 0.2% trypan blue. For this assay, we incubated four different O-GNR concentrations (10 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1} and 300 μg mL^{-1}) with cells at a density of 2×10^5 in 24-well plates and incubated them for 24 h. After that, 10 μL of the cell suspension with trypan blue were read in the Countess Automated Cell Counter ™ (Invitrogen®) to exclude counting dead cells. Data points represented in triplicate from three independent experiments. The results were analyzed using GraphPad Prism InStat software (version 6.1 San Diego, CA, USA), Two-way ANOVA, Tukey's multiple comparisons test.

2.4. Gene expression analysis

For gene expression analysis, the human osteoblast cell line MG-63 were divided in three groups: treated groups with two different O-GNR concentrations (100 μg mL^{-1} and 200 μg mL^{-1}) and control group (without O-GNR). Groups with 10 μg mL^{-1} and 300 μg mL^{-1} were excluded of this analysis due to the fact the results of viability cell for 10 μg mL^{-1} group were statistically similar to the 100 μg mL^{-1} group. In the 300 μg mL^{-1} group, was observed an expressive decrease in the number of cells showing high cytotoxicity in this concentration. The results represented triplicate from three independent experiments.

2.5. RNA extraction and synthesis de cDNA

RNA extraction using an established protocol for Trizol® Reagent (Life Technologies, Rockville, MD, USA) were performed. Cells were seeded in the same conditions described for the cellular toxicity assay and analyzed after 7 days. RNA integrity was assessed using 1.5% agarose gel electrophoresis to analyze the 18S and 28S bands. Results were quantified by ultraviolet absorption spectroscopy using NanoDrop equipment (ND-1000 Spectrophotometer v.3.0.7 - Labtrade) analyzing ratios at 280/260 and 260/230. cDNA synthesis reactions were carried out using a thermal cycler (Biocycler, MJ96G, USA) and 2 μg of RNA through a reverse transcription reaction following the manufacturer's instructions of the commercial kit ImProm-II™ Reverse Transcription System (Promega, São Paulo, Brazil). Reverse transcription (RT) was carried out for 5 min at 25 °C, 60 min at 42 °C, followed by 15 min at 70 °C and the reaction mixture was stored at −20 °C.

2.6. RT-qPCR

Five genes involved in bone repair were evaluated by RT-qPCR amplifications. *ALPL* (alkaline phosphatase), *OPN* (osteopontin), *OC* (osteocalcin), *COL I* (Collagen type I) and *RUNX2* (runt-related transcription factor 2) were performed in triplicate on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using kit GoTaq® qPCR Master Mix (Promega, São Paulo, Brasil).

The primers for amplification of five target genes and reference genes were determined using IDT software (Integrated DNA Technologies/available on: http://www.idtdna.com) and Primer-Blast software (available on: http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primers used are listed in Table 1. Data were normalized based on the expression of the reference gene. We quantified the transcripts of housekeeping genes *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *18SrRNA* (18S ribosomal RNA), and *β-actin* (actin smooth muscle-beta) and selected as endogenous control *β-actin* gene, which provided increased accuracy and resolution in the quantification of gene expression data, facilitating the detection of smaller changes in gene expression than otherwise possible. Standard PCR conditions