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CYTOTOXICITY STUDY OF GRAPHENE OXIDE AGAINST VERO LINEAGE CELLS[1]

ESTUDO DE CITOTOXICIDADE DO ÓXIDO DE GRAFENO FRENTE A LINHAGEM DE CÉLULAS VERO

Altevir Rossato Viana[2], Bruno Salles[3], Franciele da Silva Bruckmann[4], Luciana Maria Fontanari Krause[5], Sérgio Roberto Mortari[5], Cristiano Rodrigo Bohn Rhoden[6]

ABSTRACT

The Nobel Prize awarded in 2010 to researchers Andre Geim and Konstantin Novoselov of the University of Manchester push many different research groups to studying graphene, a material considered a more promising allotropic form of carbon. Graphene, according to IUPAC, consists of a sheet of carbon atoms, densely packed with a thickness of only one atom. Graphene oxide (GO) is a highly oxygenated derivative raw material. Its chemical structure and wide surface area made possible several chemical modifications and their functionalization's make of graphene oxide an excellent platform to carry magnetic nanoparticles. In order to apply in physiological studies, we investigated the cytotoxicity of graphene oxide against the VERO line at different concentrations (1, 3, 10, 30, 100, 300 µg/mL). After statistical analysis using two-way ANOVA, followed by Tukey test using GraphPad Prism 5 software. The two lowest concentrations (1 and 3µg/mL) showed no toxicity in the chosen strain and did not produce reactive oxygen species. These results may stimulate further studies for the applications of GO in biological interactions related on the adhibition of these materials in therapeutics.

Keywords: carbon, cell, treatments, toxicity.

RESUMO

O prêmio Nobel concedido em 2010 aos pesquisadores Andre Geim e Konstantin Novoselov, da Universidade de Manchester, instigou em muitos outros grupos de pesquisa o interesse em estudar o grafeno, material considerado a forma alotrópica do carbono mais promissora. O grafeno, de acordo com a IUPAC, consiste em uma folha de átomos de carbono, densamente compactados com espessura de apenas um átomo. O óxido de grafeno é um derivado da matéria prima original, mas altamente oxigenado. Sua estrutura química e sua elevada área superficial fazem com que sejam possíveis várias modificações químicas e suas funcionalizações fazem do óxido de grafeno uma excelente plataforma para carrear nanopartículas magnéticas. Para a aplicação em estudos fisiológicos investigou-se a citoxicidade do óxido de grafeno frente à linhagem VERO em diferentes concentrações (1, 3, 10, 30, 100, 300µg/mL). Após análise estatística utilizando ANOVA de duas vias, seguido pelo teste de Tukey usando o software GraphPad Prism 5, verificou-se que as duas menores concentrações (1 e 3µg/mL) não apresentaram toxicidade na linhagem escolhida, além de não produzirem

[1] Study developed at the Nanosciences Posgraduate Program.

[2] PhD student at the Nanosciences Posgraduate Program - Universidade Franciscana (UFN). E-mail: rossato.viana@hotmail.com

[3] MsD sudent at the Nanosciences Posgraduate Program - Universidade Franciscana (UFN). E-mail: brunocsalles@live.com

[4] Graduation sudent at the Universidade Franciscana. E-mail: francielebruckmann2@gmail.com

[5] Colaborators - Universidade Franciscana (UFN). E-mails: lfontanari@yahoo.com.br; mortari@ufn.edu.br

[6] Correspondent Autor: Nanosciences Posgraduate Program - Universidade Franciscana (UFN). E-mail: cristianorbr@gmail.com

espécies reativas ao oxigênio. Estes resultados poderão estimular novos estudos buscando a aplicação deste material em diferentes interações biológicas relacionadas a inclusão destes materiais e terapias associadas.

Palavras-chave: *carbono, célula, tratamento, toxicidade.*

INTRODUCTION

Nanotechnology is an interdisciplinary field of research which manipulates and controls structures from 0.1 to 100 nm and manufactures relevant materials derived from atoms and molecules (HEIDEL; DAVIS, 2011). Among the fields of nanotechnology, the production of carbon nanostructures for various applications is a field in great expansion. Examples of these structures are graphite, fullerenes, carbon nanotubes and graphene along with their precursors (SINGH *et al.*, 2018).

Graphene oxide (GO) consists of a structure analogous to the graphene structure, however oxidized. Derived from graphite chemical exfoliations, the GO comes in growing line of research due to its easy preparation and versatility of applications, considering its unique physicochemical properties and large surface area (EDWARDS, COLEMAN, 2013). Its main structural features are the abundant oxygen functional groups (carbonyls, hydroxyls, epoxide, etc.), distributed semi-randomly in their hexagonal carbon lattice. Graphene oxide is considered a promising material for biological applications because of its surface functionality and excellent aqueous processability (RHODEN *et al.*, 2017), moreover providing larger reactions sites suitable for interactions comparing to the graphene two-dimensional structure (AHMAD, 2018).

Regarding their production methods, various conditions and reagents are studied for the process. Most notably, methods include oxidation with permanganate (Hummers) or chlorate (Brodie) used are mainly due to their efficacy and reproducibility (WONG, 2014). In recent years, methods of improving the production techniques have been studied in order to obtain better efficiency and better control of the morphology of the oxide obtained. Influencing directly on the reactivity of the material (SHAMAILA, 2016; LING, 2019).

The applications of GO extend to polymer composites, sensors, purification materials, among others (LWAL, 2019, AHMAD, 2018). In the manner of biological applications, several studies are directly related to the interaction of GO with cells and tissues. Acting both in the aid of drug loading and in the treatment of diseases (GOENKA, 2014; WANG, 2011). Areas such as stem cell studies, epithelial genesis, myogenesis and neurogenesis, due to their biocompatibility and selectivity (LIU *et al.*, 2011; PRIYADARSINI, 2018). Thus, there is a need for further studies involving their interaction with biological agents to understand and overcome possible toxic effects. According to the International Standard Organization, ISO 10993-1:2009, the *in vitro* cytotoxicity assay is the beginning to evaluate the biological compatibility of any material for use in medicine. Therefore, the objective

of the present study was to evaluate the cytotoxicity of graphene oxide in a non-tumor lineage by different assays, and to verify if it produces reactive oxygen species in cell culture.

MATERIAL AND METHODS

Graphene Oxide Synthesis

The synthesis of graphene oxide (GO) was carried out by adaptation in the method of Hummers and Offeman (1958). A mixture containing 5 g of graphite (Sigma Aldrich®) and 5 g of sodium nitrate (Synth®) was oxidized using 200 mL of a concentrated solution of nitric acid 65% (Quimex®) and sulfuric acid 96% (Synth®) (1:3 ratio) and kept in an ice bath under magnetic stirring. Then, 2.5 g of potassium permanganate 99% (Vetec®) was slowly added under vigorous stirring for 2 hours. Afterwards, the solution was stirred for 30 minutes, maintaining the temperature at 60 °C and 40 mL of hydrogen peroxide (30%, v/v) was slowly added under magnetic stirring. Subsequently, 50 mL of hydrochloric acid 37% (Synth®) (10%, v/v) and 450 mL of purified water were added, and the mixture was then kept in a refrigerator at 4 °C for 24 hours. The light brown supernatant was collected, and the graphene oxide was separated by centrifugation and dried in the desiccator for 24 hours furnishing 7.4 g of GO (RHODEN *et al*., 2017).

Cell culture

VERO cells (monkey kidney) were purchased from the Rio de Janeiro Cell Bank (BCRJ). These cells were frozen in aliquots containing $1x10^6$ cells/mL fetal bovine serum (FBS) (Gibco®) plus 10% dimethyl sulfoxide (DMSO) (Synth®) in cryopreservation vials (Kasvi®). Cells were thawed carefully to decrease the effects of DMSO at room temperature. They were resuspended in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich®) and rotated for 5 minutes at 1500 rpm in a tube centrifuge (Eppendorf). The supernatant was discarded and then counted through 0.4% Trypan Blue (Sigma-Aldrich®). The pellet suspended in 1 mL of complete medium containing 10% FBS and 1% penicillin/streptomycin was placed in a 25 cm^2 polyethylene bottle (Corning®) which already contained 4 mL of the same medium. The cells were placed in a humid atmosphere containing 5% CO_2 for 72 hours, which required their peal to another 25 cm^2 bottle. After 4 passages, we had enough cells to perform the experiment.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)(MTT) assay

The colorimetric assay was performed according to Mosmann (1983). The adherent cells of the VERO strain were mechanically removed from the bottom of the polyethylene bottles with the

aid of a cell scraper (TTP). They were placed in 15 mL falcon tubes (Corning) and centrifuged for 5 minutes at 1500 rpm. After this step, the pellet was resuspended in 1 mL of complete culture medium for counting in a Neubauer chamber with the aid of trypan blue. Subsequently, a concentration of $1x10^5$ cells/mL was defined, in which 200 µL per well was added in 96-well plates containing a boring background (Corning). After 24 hours, time required for cells to adhere to the bottom of the plate, treatments containing graphene oxide (GO) at concentrations of (1, 3, 10, 30, 100, 300 µg/mL) were added same step repeated in the other essays, in addition to the negative control (+ culture medium) and positive control (cells + culture medium + H_2O_2). After 24 hours of the cells containing the treatments mentioned above, 20 µL of the MTT reagent diluted in PBS (5 mg/mL) was added to the wells. The supernatant was withdrawn and 200 µL of DMSO was added to dilute the formazan crystals adhered to the bottom. Subsequently, was performed the reading on an ELISA plate reader at a wavelength of 570 nm. (LI; SONG, 2007).

3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral red assay

The neutral red method was originally described by Borenfreund and Puerner (1985). The Neutral Red technique measures cell viability based on lysosomal activity. Viable cells with their working lysosome retain Neutral Red within these structures (TRIGLIA *et al.*, 1991).

2‘,7‘-*Dichlorofluorescein* (DFCH-DA)

The reagent used in this test, DCFH-DA is a non-fluorescent probe that easily crosses the cell membrane, thus allowing this assay to be performed (BASS *et al.*, 1983). DCFH-DA is a method based on the use of the 2’-7’-dichlorofluorescein diacetate probe (DCFH-DA), forming 2 ‘, 7’-dichlorodihydrofluorescein (DCFH). This non-fluorescent metabolite, when in contact with ROS, turns into dichlorofluorescein (DCF), which emits fluorescence (CHEN *et al.*, 2010).

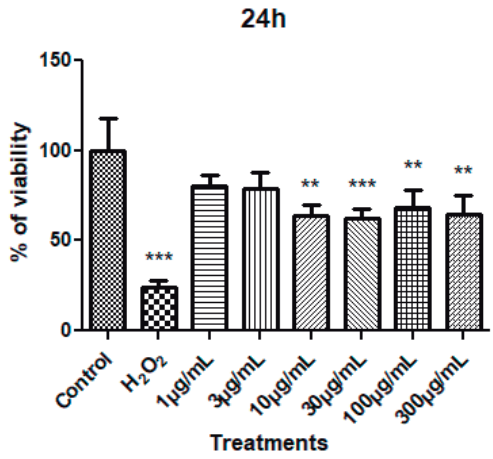
Statistical analysis

Analyses were done using two-way ANOVA, followed by the Tukey test using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences with $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** to display the different levels of significance, such as Sadegui *et al.* (2019).

RESULTS

Colorimetric viability assay (MTT) from cell dehydrogenases shown in Figure 1 below:

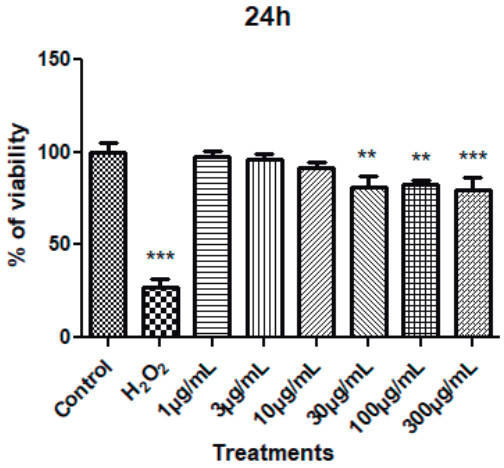
Figure 1 - GO cytotoxicity assay in VERO cells.



After 24 hours of GO exposure in MTT assay, VERO cells showed a significant toxicity at 10, 30, 100 and 300µg / mL. This result was not demonstrated in the two lowest concentrations when compared to the negative control (cells + culture medium).

Colorimetric viability assay from the neutral red vital dye shown in Figure 2:

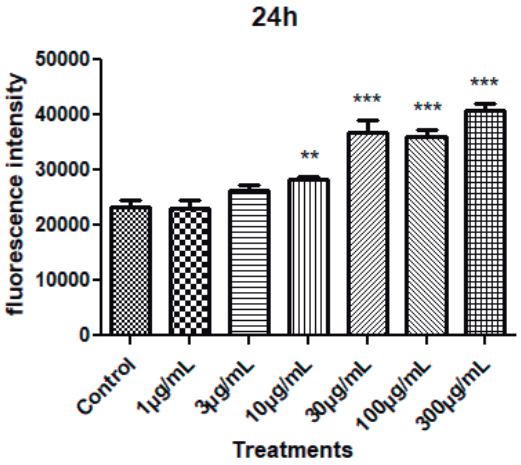
Figure 2 - GO cytotoxicity assay in VERO cells.



After 24 hours of GO exposure in Neutral Red assay, the 3 highest concentrations used presented significant cytotoxicity in relation to the control negative.

Fluorimetric conversion test of the non-fluorescent dichlorofluorescein diacetate compound (DCFH-DA) to the 2'-7 'dichlorofluorescein fluorescent compound shown in Figure 3 below:

Figure 3 - GO production of reactive oxygen species in VERO cells.



The concentrations of 10, 30, 100 and 300 µg / mL presented high production of reactive oxygen species in relation to the control without treatment after 24 hours of GO exposure.

DISCUSSION

In this study, we evaluated the synthesis of GO and its cytotoxicity in a VERO cell, which is widely applied in *in vitro* assays. Cytocompatibility is an essential requirement for new biomaterials (YUE *et al*., 2019). The results demonstrated that low concentrations showed no decrease in cell viability in comparison with the negative control.

In relation to the cytotoxicity presented in this study, concentrations of 1 and 3 µg/mL were shown to be safe in the MTT assay, however Yang *et al.* (2018) reported that GO was cytotoxic in a concentration level of 1µg / mL in cells RAW264.7, and cells in necrosis starting at 8 µg/mL. However, in the study conducted by Yang *et al.* (2016), they achieved through graphene oxide a stimulus for neurogenesis in nerve cells from in vitro assays, being a promising platform for use in neurodegenerative diseases and mental disorders. Another study by Yang and collaborators (2018) shows the biocompatibility of GO, showing a very low levels of bone cells in apoptosis and necrosis analyzed with a flow cytometer kit in contact with this nanostructure for 72h.

Nanda, Seong, and Dong (2015) demonstrate the decreasing of GO toxicity when conjugated with cysteine. All concentrations tested at 50, 100, 150, 200, 250, and 300 µg / mL did not show cytotoxicity by the MTT assay in murine cell lines SCC7. Wang *et al.* (2013) tested GO on human lung cells (HLF), observing that the concentrations of 1 and 10 µg / mL did not decrease viability of this lineage, unlike the two higher concentrations of 50 and 100 µg / mL than after 24 hours proved to be toxic.

In the study by Lammel *et al.* (2013), the researchers found no toxicity of GO produced by them up to a concentration of 16µg / mL in the neutral red assay. As in our study, the GO was to

present toxicity in this same test only at concentrations above 30µg / mL. Syama *et al.* (2017) collected primary mouse cells and were also exposed to GO for a 24-hour period, showing a production of reactive species but no cell death through the same assays performed in the present work.

Jiang *et al.* (2015) used different types of GO, but only one showed no increase in reactive species levels of 5µg/mL, a concentration very close to the samples in our study that also did not increase fluorescent DCF production. In contrast to our study showed an increase in reactive oxygen species by 30 µg/mL, the concentration of 75 µg/mL was sufficient to increase 2'-7' dichlorofluorescein fluorescent levels, most likely because they are non-tumor cells different Saos-2, MC3T3-E1 and RAW-264.7 after 24 hours of exposure (MATESANZ *et al.*, 2012).

Considering these results, the toxicity of the graphene is directly confirmed in its size, presence of functional groups, stages after the application of dispersion in the aqueous medium, variable variables (LALWANI *et al.* Al., 2016). Cell damage by reactive oxidation species has been postulated as a primary mechanism of graphene cytotoxicity (JASTRZEBSKA; OLSZYBA, 2015). Studies demonstrated that graphene sheets with live parts can also induce physical damage and interact with phospholipids, causing membrane destabilization (LALWANI *et al.*, 2016). Different mechanisms by which graphene derivatives enter the cell have been proposed, including energy independent diffusion (Raffa *et al.*, 2008) and energy dependent endocytosis (Porter *et al.*, 2007). It is important to observe that different intracellular locations of NMs may lead to different intracellular destinations and toxicity (KALMAN *et al.*, 2019).

FINAL CONSIDERATIONS

According to the results of the present study, graphene oxide demonstrates no cytotoxicity at the lowest concentrations tested. These results may indicate a possibility of applications of this material in different biological protocols. However, further studies are necessary to verify and overcome the toxicity in the highest concentrations to achieve the desired biocompatibility in non-tumor cells. Perhaps coating with a compatible polymer that does not have the same interaction and/or accumulation in the cells.

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