Interlab study on nanotoxicology of representative graphene oxide

Nelson Durán[1,2,*], Diego S.T. Martinez[2,3], Giselle Z. Justo[4], Renata de Lima[5], Vera Lúcia de Castro[6], Gisela A. Umbuzeiro[7], Edison Barbieri[8], Marcela Durán[1,11], Patricia S. Melo[9], Oswaldo L. Alves[2,10], Wagner J. Fávaro[2,11]

[1]Chem. Inst (IQ). Universidade Estadual de Campinas (UNICAMP), SP, Brazil.
[2]NanoBioss, IQ-UNICAMP, SP, Brazil.
[3]Braz. Nanotechnol. Nat. Lab. (LNNano), SP. Brazil.
[4] Fed. Univer. São Paulo (UNIFESP), SP, Brazil.
[5]Univer. Sorocaba (UNISO)/Biotechnol, SP, Brazil.
[6]EMBRAPA/Lab. Ecotoxicol. Biosaf. SP, Brazil.
[7]Fac. Tech.. Ecotox. Environ. Microbiol. Lab., UNICAMP,SP, SP, Brazil.
[8]Fisheries Inst. São Paulo, Ecotox. Lab., SP, Brazil.
[9]Biochem-METROCAMP, SP, Brazil.
[10]Chem. Inst., Solid Chem. Lab. IQ-UNICAMP, SP, Brazil.
[11]Depart. Struct. Funct. Biol. IB-UNICAMP, SP, Brazil.

duran@iqm.unicamp.br

Abstract. The graphene sample GO:Single-layer graphene oxide, purity 99%, thickness 0.7-1.2 nm (AFM); ~300-800nm X&Y dimensions is the standard size <450 nm & 1-20 μm lateral dimensions. Cheap Tubes Inc., Bratleboro, USA was selected for our study. Exhaustive characterization of GO was afforded. It exhibited thermal stability over 60°C and it was suspended in deionized water after ultrasonication (1 mg/mL) (stable 10 days). All the biological fluids used in the different assays were used as control of the colloidal suspension stability. Then, all the studies were carried out within that stability period. The cytotoxicity assays were carried out by the Alamar Blue (Resazurin) reduction, MTT and flow cytometry assays in mouse embryonic fibroblast cells (3T3), human keratinocytes (HaCaT), colorectal cancer cells (Caco-2/HCT 116), Lewis lung cancer cells (3LL), acute myeloid leukemia cells (KG-1, Jurkat, Kasumi-1) and chronic myeloid leukemia cells (K562, Lucena) and no significant toxicity was found after exposition to 0.1-100 μg/mL for 24 and 48 h. Breast cancer cells, MCF-7, showed a 20% reduction on cell viability at 24 and 48 h. No cytotoxicity were found in lymphocytes, Chinese hamster ovary cells (CHO) and human macrophage cell line (U937) at 0.1-50 μg/mL, but 30-50% survival inhibition was observed at 100 μg/mL. A dose-dependent increase in apoptosis was observed in some cells (Kasumi-1, Jurkat and K562 cells). In the case of CHO and 3T3 cells, greater levels of necrosis with increasing concentrations of GO (>50 μg/mL) were observed. Genotoxic study using the Comet assay showed slight DNA damage in lymphocytes at all concentrations tested, while more significant effects was observed in CHO cells. Econanotoxicity was carried out by lethality assays in the nematode *Caenorhabditis elegans*,d in the freshwater coelenterate *Hydra*, *Daphania* amd in Shrimp with no signs of toxicity at concentrations varying from 0.1-100 μg/mL of GO. However, death and disintegration of *Hydra* was observed after exposition to 100 μg/mL for 72 h. In *in vivo* studies, no changes in biochemical parameters of Fischer 344 rats were observed after the i.p. administration of GO. Some black agglomerates were found in the intraperitoneal cavity of rats injected with GO. However, in Fisher 344 rats-bearing prostate tumors, treatment with GO (up to 100 μg/mL) negatively affected the hepatic parameters, whilst in the renal ones, an improvement was observed. Studies are in progress to understand the mechanisms involved in the uptake of GO by RES. GO appears as a potential non-toxic *in vitro* and *in vivo* assays at the concentrations used in this interlab experiments.

1. INTRODUCTION

Graphene oxide (GO) is a material with potential in many different applications and with thermal stability [1]. GO is not considered till now as representative compound in the actual studies in the EU (e.g. REACH, OECD). Recently, European Commission's JRC Institutes IRMM (Belgium) and IHCP (Ispra, Italy) introduced the term 'representative test material' (RTM), and provided a frame for its use [2]. The definition of RTM is a material from a single batch, homogeneous and stable with respect to one or more specified properties (ISO/AWI TS 16195). The ISO Tech. Comm. Nanotechnol. (TC 229) has recently published a new standard: ISO/TR 16197:2014 – Nanotechnologies – Compilation and description of toxicological screening methods for manufactured nanomaterials. This standard is a Technical Report related to *in vitro* and *in vivo* methods that can be useful for the toxicological and ecotoxicological measurement, complementing the ISO/TS 27687:2008 and ISO/TS 80004-1. In view of these documents, our Brazilian Network on Nanotoxicology-GIGENANOTOX (MCTI/CNPq) decided to follow and evaluate these new rules for a selected representative compound, GO, by different nanotoxicological assays.

2. MATERIAL AND METHODS

2.1. Sample

The graphene sample GO:Single-layer graphene oxide, purity 99%, thickness 0.7-1.2 nm (AFM); ~300-800nm X&Y dimensions is the standard size <450 nm & 1-20 µm lateral dimensions from Cheap Tubes Inc., Bratleboro, USA was selected for our study. Exhaustive characterization of GO was afforded by spectroscopic and microscopies techniques..

2.2. Debris determination in GO

To isolate oxidation debris fragments, a suspension of sample raw-GO (0.5 mg mL^{-1}) was produced through sonication for 30 min in an ultrasound bath. The suspension was brought to reflux in a 0.1 mol L^{-1} NaOH solution for 90 min, but resulting in a light supernatant and a black pelletized sediment. The black pellet was separated from the supernatant by filtration, reprotonated with a 1.0 mol L^{-1} HCl solution, dialyzed, and finally dried by lyophilization thus yielding a black powder (sample df-GO: debris-free graphene oxide)[3].

2.3. Colloidal stability of GO in different biological media used;

The standard ASTM E2524-08 (Standard test method for analysis of haemolytic properties of nanoparticles) was followed. It was added 1.5 mL of an stock GO solution (1 mg/mL) to 13.6 mL of each culture medium or water from different sources in Falcon tube (15 mL). It was transferred 1 mL of these solution to Epperdorf tubes, in order to visualize the experiment. The time of observation was 0-120 h. An aliquot of 200 µL of each samples collected from these experiments were transferred to plate with 96 wells and the absorption was measured at 450 nm in the ELISA protocol. At the same time it was photographed all the eppendorf tubes at the time interval of 6 and 144 h.

2.4. Hemolysis

The hemolytic assay was carried out following the standard procedure from ASTM E2524-08 (Standard test method for analysis of hemolytic properties of nanoparticles.

Hemolytic activity assay was performed according to our earlier report [4]. Briefly, fresh human blood (volunteer at ethical conditions) was collected in a centrifuge tube containing anticoagulant, trisodium citrate (3.2%), and was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and only the erythrocytes were collected. Erythrocytes were further washed three times with PBS (pH = 7.4). A

10% (v/v) suspension of erythrocytes in PBS was prepared and 1.9 mL of this erythrocyte solution was placed in a 2 mL centrifuge tube and 0.1 mL of GO)Stock solution 1 mg/mL) (up to 100 µg/mL) in PBS (pH 7.4) was added to it. The tubes were then incubated for 1h at 37^{o}C. For comparison, Triton X-100 (0.2%) and PBS were taken as the positive and negative controls, respectively. After incubation the tubes were centrifuged at 3000 rpm for 10 min, and absorbance of the supernatant was taken at 570 nm in UV-visible spectrophotometer.

2.5. Cytotoxicity

The cytotoxicity assays were carried out by the Resazurin reduction, MTT and flow cytometry assays in mouse embryonic fibroblast cells (3T3-ATCC CCL-163), human keratinocytes (HaCaT-Amsterdam Medical Center, The Netherland)), colorectal cancer cells (Caco-2-ATCC HTB-30)/HCT 116-ATCC CCL-247), Lewis lung cancer cells (3LL-ATCC CRL-1642), acute myeloid leukemia cells (KG-1-ATCC CCL-246, Jurkat-ATCC TIB-152, Kasumi-1-ATCC CRL-2724) and chronic myeloid leukemia cells (K562-TCC CCL-243, Lucena-Inst. Medical Biochemistry-UFRJ, Brazil), breast cancer cells (MCF-7-ATCC HTB-22), Chinese hamster ovary cells (CHO-K1-ATCC CCL-61), breast cancer cells (SKBR3-ATCC HTB-30) and human macrophage cell line (U937-ATCC CRL-1593.2). All the cell were checked for mycotoxin by PCR and Flouorescence assays.

2.6. Genotoxicity

The genotoxicity of GO was studied using 3T3, CHO-K1 and Jurkat cells. In brief: The lymphocytes, CHO-K1 and 3T3 cells were exposed to the GO suspension (stock solution 1 mg/mL) for a period of 1 hour. Negative and positive controls employed phosphate buffered saline (PBS) and H2O2 (0.1 mM), respectively. The comet assay was performed as described by Azqueta et al. [5]. Lymphocytes: Each treatment used 10 µL of lymphocytes in 110 µL of low melting point agarose (0.8%), and the mixture was placed onto microscope slides that had been pre-coated with normal melting point agarose (1.5%). After polymerization, the cover slips were removed and the slides were treated with an ice-cold lysis solution for 20-30 minutes. All treatments were then incubated in an electrophoresis buffer for 20 minutes, followed by electrophoresis for 20 minutes at 1.3 V/cm. After electrophoresis, the slides were stained and analyzed by optical microscopy. Scores of between 0 and 4 were assigned according to the quantity of DNA in the tail and the length of the tail: Score 0 corresponded to intact cells, with no damage caused by the exposure; Score 1 corresponded to cells with minimal damage; Score 2 to average damage; Score 3 to severe damage; and Score 4 to cells with maximum damage. In this visual method, the number of cells found for each score was multiplied by the value of the score and the values were summed at the end of the analysis of each slide. Since the score depended on the number of cells observed, an index of tail damage (TD) was created by dividing the score given for the slide by the number of cells analyzed on the slide [5, 6-8].

2.7. Tali analysis

Cell viability was measured using a Tali™ Apoptosis Kit consisting of Annexin V Alexa Fluor® 488 and Propidium Iodide (Invitrogen), and a Tali™ image-based cytometer, which enabled the numbers of viable, apoptotic, and dead cells to be counted. The cells that had been treated with SH-MNPs (both MSA- or DMSA-coated MNPs) and SNO-MNPs at nanoparticle concentrations (0.01; 0.05 and 0.1 mg/mL), nanoparticles were centrifuged and concentrated to 1 x 10^{6} cells/mL. 100 µL aliquots of sample were prepared according to the specifications of the kit, and the tests were performed in triplicate [9].

2.8. Salmonella/microsome assay testing

S. typhimurium TA98 *his*D3052, Δ(*uvr*B, *bio*), *ffa*, Ap^{r}; frameshift mutation, pKM101 was used for these experiments, following the OECD 471 standard from OECD. It is based upon scoring bacterial

growth (colonies) on selective agar plates after exposure of the bacterial cells to a test chemical, either by incorporation of the test chemical into the agar plates, or by pre-incubation prior to plating out [10].

2.9. Econanotoxicity

These experiments were carried out by lethality assays in the nematode *Caenorhabditis elegans* and in the freshwater coelenterate *Hydra*.

2.9.1. C. elegans*:*

2.9.1.1. Maintance of the C. elegans cutures **:** Strain N2 was purchased from *Caenorhabditis* Genetic Center (Brazil) following the standards procedures[11]. The nematodes were cultivated at 20^{o}C in Petri plates in nematodes medium (NGM) seeding with *E. coli* strain OP50 for feeding. To obtain synchronized cultures, pregnant hermaphrodites nematodes were lysed in an alkaline hypochlorite solution. All the assays with the nematodes were carried out in a liquid medium (S-Medium or M9) using plates with of 24 or 96 wells.

2.9.1.2. Lethality assay: This measure was evaluated by alive nematodes. After exposure of to GO for 72 h at 20^{o}C, the nematodes were observed in an inverted microscope and classified as dead or alive (n = 120 nematodes). Each assay consisted in seven different concentrations of GO (0.1-100 μg/mL) and one as control (triplicated assays).

2.9.1.3. Growth **:** The growth was evaluated by the nematode body length. He body length was determined usinf the plane surface of the nematodes using a software Axiovision ZEISS (n = 50 nematodes)

2.9.1.4. Statistic analises estatística **:** For this analysis was used the GraphPad Prism 5.0 program (La Jolla, CA, USA). To verify the occurrence of significant differences between the study groups was used in the experiments analysis of variance (ANOVA). Where significant differences were found, the Tukey test was used to test existing minor differences between all groups. To assess significant differences between two groups, Student's t test was used unpaired. In all analyses, α was 5% ($p < 0.05$).

2.9. 2. Hydra: When exposed to toxicants, hydras can display a broad range of morphological changes indicative of either sub lethal or lethal effects. Increasing degrees of intoxication departing from normalcy are characterized by stages showing clubbed tentacles, shortened tentacles and the ‘‘tulip phase’’, which leads to the death of the organism.

Hydra attenuata (Cnidaria) was cultured in hydra medium (TES). It was fed 3 times per week with *Artemia nauplii*. Cultures were maintained at controlled conditions of temperature and light. Toxicity tests were carried out in microplates to which the GO will be added. Hydras were exposed to samples for 96 h. Three nonbudding hydra will be selected to be exposed to each concentrations tested. Seven test concentrations (0.1 μg/mL; 1.0 μg/mL; 5.0 μg/mL; 10 μg/mL; 25 μg/mL; 50 μg/mL and 100 μg/mL) and a control were used. The progressive effects of the GO concentrations were recorded daily by rating the morphological status of the individual polyp and assigning a score from 10 if normal to 0 if disintegrated [12, 13]. Median scores of progressive responses were analyzed at each exposure time for each GO tested concentration.

2.9.3. Daphnia acute toxicity test (48h).

Daphnia similis Straus 1820 (Crustacea, Cladocera) was used following the OECD guideline [14]. Young daphnids, aged less than 24 h at the start of the test, are exposed to the GO at a range of concentrations for a period of 48 hours. Immobilization is recorded at 24 h and 48 h and compared with control values. The results were analyzed in order to calculate the EC50 at 48h.

2.9.4. **Acute ecotoxicity on freshwater Shrimp**

On this experiment 60 shrimps (*Palaemon pandaliformis*) were used. The shrimp specimens were acclimatized in the salinity of 30%to 20ºC with constant aeration, cleaning and daily renewal of the water for a period of 5 days prior the experiments. Before starting the experiment, the animals were

kept individually in respirometric tubular chambers with continuous water circulation for 60 min in order to minimize the stress caused by handling. At the end of the acclimation period GO was separately introduced in an amount determined in respirometers with a precision pipette in order to achieve the desired final concentration. The difference between the concentrations determined at the beginning and end of the confinement (after 30 minutes) were used to calculate the oxygen consumption (mL/g/h) and ammonia excretion (µl/mL/h) taking into account the respirometer volume and the animal's wet weight. The dissolved oxygen was determined through the Winkler method and the ammoniacal nitrogen based upon the phenol hypochlorite method. Average oxygen specific consumption and ammonia excretion by shrimp were evaluated through variance analysis [15].

2.9.5. The Ames Salmonella/microsome mutragenicity assay

Salmonella typhimurium TA98 *his*D3052, Δ(*uvr*B, *bio*), *ffa*, Ap^r; frameshift mutation, pKM101 was used for this mutagenic study. It was followed the OECD 471 Bacterial Reverse Mutation Test, OECD, Paris, France (1997) standard procedure [10].

2.10. In vivo rats toxicity study

2.10.1. Genotoxicity: Micronucleous assay **[16, 17]**.

A total of 25 rats (Fischer 344) were divide in 5 groups (n = 5 animals): negative control group (NCT): 0.3 mL of physiological solution (0.9% intraperitoneal (i.p.) administration; Positive control group (PCT): dose of 1.5 mg/mL of *N-metil-N-nitrosouréia* (MNU - *Sigma, St. Louis, MO, EUA*) by i.p. administration; GO group-1 (GO 1): dose of 0.5 mg/mL of GO by i.p. administration; GO group-2 (GO 2): dose of 5 mg/mL of GO by i.p. administration; GO group-3 (GO 3): dose of 25 mg/mL of GO by i.p. administration.

Animals were euthanized 24 hours after the administration of chemical agents. Femurs were removed and cleaned; and the epiphysis was sectioned to expose the spinal canal. The bone marrow was aspirated with 1 mL fetal bovine serum (FBS) and placed in 2 ml Falcon tube containing FBS. The bone marrow was suspended several times to form a suspension with FBS. Subsequently, the samples were centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the precipitate was carefully aspirated with a pipette. Next, 100 µL of the precipitate of each sample was applied to histological slide and smear done with the aid of the cover slip. The smears were dried for 24 hours at room temperature. After this period, samples were fixed in absolute methanol for 15 min and stained with Giemsa (1:10 dilution in sodium phosphate buffer pH 6.8).

Samples were analyzed under a light microscope with 100x oil immersion objective and the number of micronuclei was counted in 3000 polychromatic erythrocytes (PCEs) per animal. The identification of micronuclei followed the criteria described by Fenech [18].

***2.10.2. Toxicological and biochemical analyses*:** It was used 20 rat (Fischer 344) and divided in four groups (n = 5 animals/group). The treatment will be exactly the same as above for four weeks. After 4 weeks of treatment, the animals were euthanized and blood samples were collected by cardiac puncture (left ventricle). The serum was separated by centrifugation at 6000 rpm, 5 min. and used to investigate the hepatotoxic effects, nephrotoxic, cardiotoxic of different concentrations of GO. This was measured through the analysis of alanine aminotransferase (ALT) that is for specific marker activity for hepatic damage; activity of aspartate aminotransferase (AST), indicative marker of liver and heart damage and circulating levels of urea and creatinine to check kidney function. Readings were performed by microplate reader Multi-Mode Microplate Reader Synergy H1M (Bio-Tek Instruments, USA) with temperature control and analyzed by Kcjúnior software (Bio-Tech Instruments, Inc., Winooski, VT, USA). For the analysis of systemic toxicity of different GO concentrations fragments of different organs were collected: pancreas, spleen, lungs, heart and kidneys; of all animals in each experimental group. Different organs were fixed in Bouin solution for twelve hours. After fixation, the fragments were washed in 70% ethanol, with subsequent dehydration in an ascending concentration of ethanol.

Subsequently, the fragments were diaphanized in xylene for 2 h and included in a polymers (Paraplast Plus, ST. Louis, MO, USA). Then the materials were sectioned in a Leica RM 2165 rotary microtome (Leica, Munich, Germany) with a thickness of 5 μm and stained with hematoxylin-eosin and photographed on Zeiss Axiophot light microscope (Zeiss, Munich, Germany).

2.10.3. Histopathology: Histopathology of various organs will be evaluated and correlated with the toxicity levels of inflammation. The degree of inflammation will be assessed by a semi-quantitative scale: 0 = no inflammation, 1 = minimal inflammation (less than five lymphocytes in an area of 0.25 mm^2), 2 = moderate inflammation (scattered throughout the tissue mononuclear inflammatory cells but stroma still was visible), 3 = intense inflammation (mononuclear inflammatory cells infiltrating tissues deeply [19].

3. RESULTS AND DISCUSSION

The graphene oxide sample GO from Cheap Tubes Inc. (USA) was selected for our study. Exhaustive characterization of GO was afforded. Characterization through XRD, XPS, NMR, Raman, FTIR, UV-Vis, TG, DSC, TEM, EDS, DLS and NTA, showed that these analyses corresponded to a single-layer graphene oxide, purity 99%, without Debris and it was stable in water suspension (1 mg/mL) for 10 days. From these analyses was clear that the representative GO from a commercial source it is single layer and a good sized distribution and easily suspended in water. From this analysis it was clear that the representative GO from a commercial source it is single layer and a good sized distribution.

It exhibited thermal stability over 60°C and it was suspended in deionized water after ultrasonication (1 mg/mL) (stable 10 days). All the biological fluids used in the different assays were used as control of the colloidal suspension stability (Fig.1).

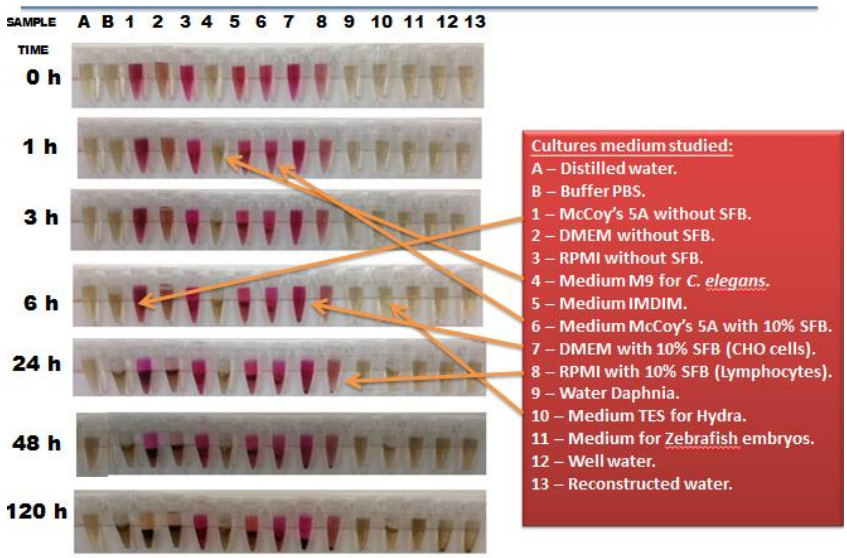

Figure 1. Colloidal stability of GO (1 mg/mL) in different biological media used in the different toxicological assays.

The Fig. 1 shows that in different media the GO showed a short period of stability. In general it was suggested for all the team that used the suspension freshly prepared during the 1-3 h period. The same results were corroborated by the UV-Vis spectroscopy. Then, all the studies were carried out within that stability period.

The hemolytic assay following the procedure from ASTM E2524-08 showed completely absence of hemolysis by GO up to 100 ug/mL,

The cytotoxicity assays were carried out by the Alamar Blue (Resazurin) reduction, MTT and flow cytometry assays in mouse embryonic fibroblast cells (3T3), human keratinocytes (HaCaT),

colorectal cancer cells (Caco-2/HCT 116), Lewis lung cancer cells (3LL), acute myeloid leukemia cells (KG-1, Jurkat, Kasumi-1) and chronic myeloid leukemia cells (K562, Lucena) and no significant toxicity was found after exposition to 0.1-100 μg/mL for 24 and 48 h. Breast cancer cells, MCF-7, showed a 20% reduction on cell viability at 24 and 48 h. No cytotoxicity were found in lymphocytes, Chinese hamster ovary cells (CHO) and human macrophage cell line (U937) at 0.1-50 μg/mL, but 30-50% survival inhibition was observed at 100 μg/mL (Table 1).

Table 1. Viability of cell cultures on graphene oxide at 24h

Cell Culture	Characteristics	MTT assay	Comments	Resazurin	Comments
3T3	Mouse embryonic fibroblast cells	Up to 50 ug/mL no toxicity	>100 ug/mL 20% inhibition	Up to > 100 ug/mL no toxicity	
HaCat	Human keratinocytes			Up to 50 ug/mL no toxicity	
Caco-2	Colorectal adenocarcinoma cells			Up to > 100 ug/mL no toxicity	
HCT 116	Colorectal carcinoma cells			Up to > 100 ug/mL no toxicity	
3LL	Lewis lung cancer cells			Up to > 100 ug/mL no toxicity	
KG-1	Acute myeloid leukemia cells			Up to > 20 ug/mL no toxicity	>80 ug/mL 15% inhibition
Jurkat	Acute myeloid leukemia cells	Up to > 100 ug/mL no toxicity		Up to > 100 ug/mL no toxicity	
Kasumi-1	Acute myeloid leukemia cells	Up to 50 ug/mL no toxicity	>100 ug/mL 20% inhibition	Up to 50 ug/mL no toxicity	>80 ug/mL 20% inhibition
K562	Chronic myeloid leukemia cells	Up to 50 ug/mL no toxicity	>100 ug/mL 50% inhibition	Up to > 100 ug/mL no toxicity	
Lucena	Chronic myeloid leukemia cells			Up to > 60 ug/mL no toxicity	>80 ug/mL 10% inhibition
MCF-7	Breast cancer cells			Up to 50 ug/mL no toxicity	>80 ug/mL 10% inhibition
SKBR3	Breast cancer cells			Up to 20 ug/mL no toxicity	>50 ug/mL 40% inhibition
CHO	Chinese hamster ovary cells	Up to 50 ug/mL no toxicity	>100 ug/mL 70% inhibition		
U937	Human leukemic monocyte lymphoma cell			Up to 30 ug/mL no toxicity	>40 ug/mL 50% inhibition
Lympho-cytes	Peripheral blood	>25 ug/mL 50% inhibition			

Note: All he cells were checked out for mycoplasma by the PCR and fluorescence assay. All the cell cultures were from ATCC. Similar results at 48 h.

In some cases there are differences in the cell sensibilities when is used Alamar Blue or MTT assays. But in general, at different laboratory the results of cytotoxicity is within the experimental errors when it was used the same cytotoxicity assay. For example, in 3T3 and K562 the MTT assay was more sensible than Resazurin assay. However, aImost identical results in the Jurkat and Kasumi cell cultures in the MTT and Resazurin assays were observed.

A dose-dependent increase in apoptosis was observed in some cells (Kasumi-1, Jurkat and K562 cells). In the case of CHO and 3T3 cells, greater levels of necrosis with increasing concentrations of GO (>50 μg/mL) were observed (Table 2).

Table 2. Graphene oxide (GO) necrosis and apoptosis

Cell Culture	Characteristics	MTT assay	Comments	Cell Death
Lymphocytes	Peripheral blood	>25 ug/mL 50% inhibition		Apoptosis
Kasumi-1	Acute *myeloid leukemia* cells	Up to 50 ug/mL no toxicity	>100 ug/mL 20% inhibition	Apoptosis
K562	Chronic myeloid leukemia cells	Up to 50 ug/mL no toxicity	>100 ug/mL 50% inhibition	Apoptosis
3T3	Mouse embryonic fibroblast cells	Up to 50 ug/mL no toxicity	>100 ug/mL 20% inhibition	Necrosis
CHO	Chinese hamster ovary cells	Up to 50 ug/mL no toxicity	>100 ug/mL 70% inhibition	Necrosis

Genotoxic study using the Comet assay showed slight DNA damage in lymphocytes at all concentrations tested, while more significant effects were observed in CHO cells.

Econanotoxicity was carried out by lethality assays in the nematode *Caenorhabditis elegans,* in the freshwater coelenterate *Hydra attenuate, Daphnia similis* and on S*hrimp*.

Chronic GO exposure on survival of *Caenorhabditis elegans* (N2 strain; 120 nematodes) at L4 stage expressed as the percentage of control viability (100%) showed no toxicity up to 100 ug/mL. The study on GO on survival (120 nematodes) and body length (50 nematodes) of *Caenorhabditis elegans* (N2 strain) at L1 stage after 72 h of exposure at 20^{o}C showed also no toxic effect in this stage. No effect up to 100 μg/mL of GO acting on *Daphnia similis* was found. Lethality assays in the freshwater coelenterate *Hydra* with showed no signs of toxicity at concentrations varying from 0.1-50 μg/mL of GO. At 50 mg/mL of GO, the tentacles was shortened after 72 h of exposure. However, death and disintegration of *Hydra* was observed after exposition to 100 μg/mL for 96 h.

In the Salmonella/microsome mutagenicity assay using 10-250 μg/plate no effect was observed.

No acute ecotoxicity was verified up to 3.0 μg/mL (Exposure time: 24h) to shrimp. Absence of effects on oxygen consumption and ammonia excretion up to 1.0 μg/mL (Exposure time: 24h)

In *in vivo* studies, no changes in biochemical parameters of Fischer 344 rats were observed after the i.p. administration of GO. Some black agglomerates were found in the intraperitoneal cavity of rats injected with GO. This was also observed previously in Balb/c mice receiving GO i.p. [20]. This probably did not affect the final absorption of GO via intraperitoneal administration, since in another experiment with this GO (100 μg/mL) on Fisher 344 rats-bearing prostate tumors, treatment with GO negatively affected the hepatic parameters, whilst in the renal ones, an improvement was observed. Studies are in progress to understand the mechanisms involved in the uptake of GO by RES.

4. CONCLUSIONS

Finally, through all of these experimental assays, such as cytotoxicity, genotoxicity, mutagenicity, ecotoxicity and *in vivo* toxicity assays, the GO appeared as a potential new drug (~50-100 μg/mL) for several diseases *in vivo* treatment with very low limitations.

Acknowledgement: Support from the Brazilian Network of Nanotoxicology (CIGENANOTOX) (MCTI/CNPq), INOMAT (MCTI/CNPq), NanoBioss (MCTI) and FAPESP are acknowledged.

References

[1] Zhou and Bongiorno A 2013 *Sci. Rep.* **3** 2484
[2] Roebben, G, Rasmussen K, Kestens V, Linsinger T P J, Rauscher H,Emons H and Stamm H 2013 *J. Nanopart. Res.* **15** 1455
[3] Faria A F, Martinez D S T, Moraes A C M, Costa M E H M, Barros E B, Souza Filho A G, Paula A J and Alves O L 2012 *Chem. Mater* **24** 4080
[4] Durán N, Makita Y and Innocentini L H 1979 *Biochem. Biophys. Res. Commun.* **88** 642
[5] Azqueta A, Gutzkow K B, Brunborg G and Collins A R 2011 *Mutat. Res.* **724** 41.
[6] Dusinská M, Collins A, Kazimírová A, Barancoková M, Harrington V, Volkovová K, Staruchová M, Horská A, Wsólová L, Kocan A, Petrík J, Machata M, Ratcliffe B and Kyrtopoulos S 2004 *Mutat. Res.* **553** 91
[7] Dusinska M and Collins A R 2008 *Mutagenesis* **23** 191
[8] Collins A R, Oscoz A A, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith C C and Stetina R 2008 *Mutagenesis* **23** 143
[9] Lima R, Feitosa L O, Ballottin D, Marcato P D, Tasic L and Durán N 2013 *J. Phys. Conf. Ser.* **429** 012020
[10] OECD 1997 Guideline 471 Ames bacterial reverse mutation screening assay (Non-GLP), Paris, France
[11] Brenner S 1973 *Brit. Med. Bull.* **29** 269
[12] Wilby O K 1988 *Proc. Workshop Ass. Franc. Teratologie* 108
[13] Wilby O K and Tesh J M 1990 *Toxicol. in Vitro* **4** 582
[14] OECD 2004 Guideline for Testing of Chemicals: Daphnia sp. Acute Immobilization Test. OECD Guideline No 202
[15] Barbieri E, Bondioli, A C V, De Melo C B and Henriques, M B 2014 *Marine Freshwater Behaviour Physiol.* **47** 1
[16] Schmid W 1975 *Mutat. Res.* **31** 95
[17] Mersch J, Beauvais M M and Nagel P 1996 *Mutat. Res.* **371** 47
[18] Fenech M 2000 *Mutat. Res.* **455**, 81
[19] Offersen B V, Knap M M, Marcussen N, Horsman M R, Hamilton-Dutoit S and Overgaard J. 2002 *Br. J. Cancer* **87** 1422
[20] Yang K, Gong H, Shi X, Wan J, Zhang Y and Liu Z 2013 *Biomaterials* **34**, 2787