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Graphene oxide sheets-based platform for induced pluripotent stem cells culture: toxicity, adherence, growth and application

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Abstract. It was prepared the graphene oxide (GO) sheets by suspension of GO in ultrapure deionized water or in Pluronic F-68 using a ultrasonicator bath. Total characterization of GO sheets was carried out. The results on suspension of GO in water showed excellent growth and cell adhesion. GO/Pluronic F-68 platform for the growth and adhesion of adipose-derived stem cells (ASCs) that exhibits excellent properties for these processes. GO in water suspension exhibited an inhibition of the cell growth over 5 µg/mL *In vivo* study with GO suspended in water (100 µg/mL) on Fisher 344 rats via i.p. administration showed low toxicity. Despite GO particle accumulates in the intraperitoneal cavity, this fact did not interfere with the final absorption of GO. The AST (aspartate aminotransferase) and ALT (alanine aminotransferase) levels (liver function) did not differ statistically in all experimental groups. Also, creatinine and urea levels (renal function) did not differ statistically in all experimental groups. Taking together, the data suggest the great potential of graphene oxide sheets as platform to ACSs, as well as, new material for treatment several urological diseases.

1. INTRODUCTION

The need to find a durable material, of easy use, and which does not interfere significantly in the growth and differentiation of stem cells for the construction of a scaffold for use in urologic surgery, with the purpose of reducing infections, regeneration times and even graft rejection during reconstitution in patients with urethral structure was conducted a broad survey of information about this and came to the consensus of this project: using graphene oxide, a widely studied nanomaterials which has been presenting numerous beneficial results when in contact with the stem cells. Advanced techniques for the growth, differentiation and proliferation of mesenchymal adipose stem cells will be used, as well as the characterization of graphene oxide sheets. Detailed analysis will be performed at each stage of this project to evaluate the interference of these nanocomposites to stem cells, the mucoadhesivity and especially in vivo toxicology of these compounds involved to build the final scaffolds. There are a few *in vivo* studies with regard to the biocompatibility of graphene oxide (GO). Previously, it was developed a synthesis of thermo sensitive hydrogel system based on GO by adding an amount of Pluronic (F-126 and F-68) as a physical crosslinker, without any chemical modification of GO [1]. It was reported that the gel formation was long lasting and stable and did not show any severe chronic inflammatory response. Besides this, it was found that adipose-derived stem cells (ASCs), showed increased adhesion when grown on GO films [2]. However, there is still some controversy in terms of cell adhesion morphology when Mesenchymal stem cells (MSCs) are in contact with GO. Probably the inconsistency may be accounted for by the difference in cell types, the GO impurities, the substrates, and the manufacturing methods of GO. In view of this facts, it was used a representative GO from CheapTubes-USA (Go-single layer).

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.

2.2.Graphene oxide sheets preparation

Colloidal dispersions of individual graphene
oxide sheets in water at the concentration of 3 mg/mL were prepared with the aid of ultrasound (Fisher Scientific FS60 ultrasonic cleaning bath) in 20 ml batches. Graphene oxide paper was made by filtration of the resulting colloid through an Anodisc membrane filter (47 mm in diameter, 0.2 mm pore size; Whatman), followed by air drying and peeling from the filter [3].

2.3. In vivo rats toxicity study

***2.3.1. 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, cardio toxic 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) [4].

2.3.2. 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 [5].

2.4. Adipose-derived stem cells (ASCs)

ASCs from Hemocenter (UNICAMP) were used [6]. The extraction protocol of mesenchymal cells and cellular cultures was based on Choudhery et al. [7]. Cells from adipose tissue samples were isolated by enzymatic digestion. Briefly, 2 mL of tissue slurry was placed in a 50-mL Falcon tube and washed vigorously five times with 5 mL of phosphate-buffered saline (PBS). Cells in the wash fraction were retained. The fatty tissue was treated with an equal volume of 0.2% collagenase type 1A (Sigma) bovine serum albumin (BSA) and gentamicin at 37°C for 30 min. Complete medium (DMEM-Low glucose), 20 mL, supplemented with 10% fetal bovine serum (FBS) was added in the digested tissue to neutralize collagenase, passed through a 40-µm filter and centrifuged at 150 g for 10

min. The cells from both the wash fraction and the digested fraction were suspended in complete medium and counted using trypan blue and Turk's stains. To prevent spontaneous differentiation, cells were maintained at sub-confluent levels (70-80%) and were harvested with 0.05% trypsin-EDTA for use in subsequent experiments.

2.5. Growing stem cells in the presence of GO

Inocula were prepared standards mesenchymal stem cells obtained from our Laboratory of Human Adipose Tissue (Medicine School-UNICAMP) in DMEM (Dulbeco's Modified Eagle Medium), and the cells were incubated for 72 hours to achieve maximum log growth for the time of incubation in an incubator at 37^{o}C in 5% CO2 with 10% calf serum [8]. Thus, inoculum from the previous one was prepared a solution of stem cells through detachment of the adherent cells in the disposable petri dish (diametro150 mm) using 1 mL of trypsin (0.005%) for 3 minutes at 37^{o}C and washing with 0.1 M of PBS (phosphate buffered saline). Cell viability by Trypan Blue staining technique and counting in a Neubauer chamber was carried out. This cell solution was used to inoculate the experiments (stock: 5x10^{3} cells / mL). Stem cells were inoculated at the concentration 10^{5} cells / mL in 2 mL DMEM culture medium in petri dishes along with graphene oxide solution at different concentrations (1- 50 µg/mL) and incubated at 37^{o}C. After 24 hours, the wells were washed with the PBS (twice) and stained with Live Dead® kit and fluorescence microscopy images obtained. Other wells were leading at the same growth conditions and after 72 h were repeated the same procedure described above with ASCs.

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. Previously, it was shown that *in vitro* and *in vivo* did not exert any nanotoxic effect up to 100 µg/mL and besides these no hemolysis was also found [9].

AST and ALT levels (liver function) and creatinine and urea levels (renal function) did not differ statistically in all experimental groups after inoculation of Fischer 344 rats with GO (Table 1).

Tabela 1: Biochemical parameters of hepatic and renal functions after 24 h of i.p. administration of GO 1 (10 µg/mL) and GO 2 (100 µg/mL).

Groups	AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	*P value*
Control	51.7±2.1 a	28.7±2.1 a	29.7±1.5 a	0.27±0.06 a	$P < 0.01$
GO 1 (10 µg/mL)	53.5±2.1 a	31.0±2.8 a	31.5±0.7 a	0.31±0.06 a	$P < 0.01$
GO 2 (100 µg/mL)	53.0±2.8 a	32.5±3.5 a	27.0±2.8 a	0.26±0.03 a	$P < 0.01$

Data expressed as average ± standard deviation (*n= 03*). Two measurements followed by the same minuscule letter indicate no differences between them by the Turkey test.

In vivo study with GO suspended in water on Fischer 344 rats via intraperitoneal administration showed low toxicity, since part of them they accumulates in the intraperitoneal cavity. Similar results with GO on bald/c mice were reported previously [10] (Fig.1).

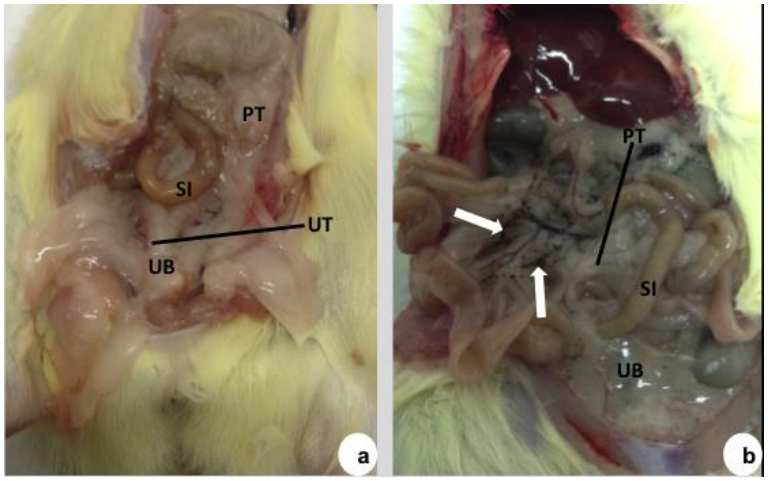

Figure 1: Exposure of abdominopelvic cavity in the animals from Control (a) and GO+Pluronic F-68 (b) groups. (a) Normal features of organs and peritoneum in the abdominopelvic cavity. (b) Presence of dark agglomerates (arrows) in the peritoneum. **a – b: PT** – peritoneum; **SI** – small intestine; **UB** – urinary bladder; **UT** – uterus

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. A significant tumor regression at those conditions was afforded [9].

In vitro cell cultures of a GO in water suspension exhibited no inhibition of the cell growth up to 5 µg/mL. A very effective cell growth occurred at 1 µg/mL (Fig. 2).

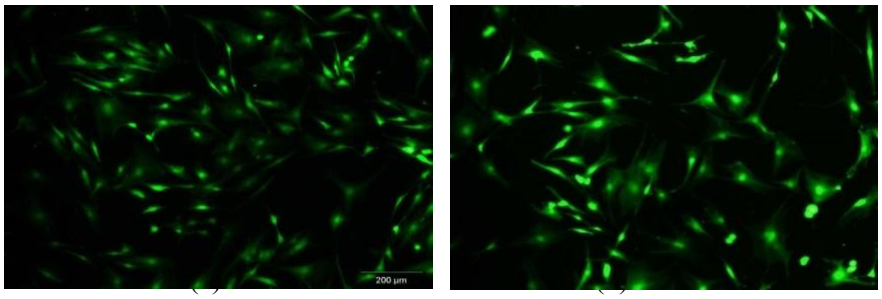

Figure 2. Fluorescence micrographs of steam cells. a).Control and b) After 24 h (GO - 1 µg/mL) (ASCs growth and adhesion).

In order to have a scaffold for ASCs it was prepared GO sheets by suspension of GO in ultrapure deionized water or in Pluronic F-68 using a ultrasonicator bath (Fig.3).

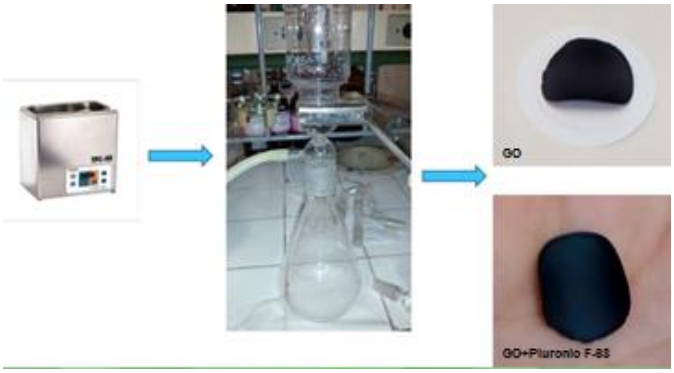

Figure 3. Preparation of GO and GOP68 sheets.

In a DMEM solution the GO shits were completely stable for several hours, in comparison that GO powder that was immediately suspended in the same medium. Preliminary studies indicate that these GO sheets led to ASCs growth without any inhibition.

4. CONCLUSIONS

GO in water suspension exhibited an inhibition of the cell growth over 5 µg/mL. GO/Pluronic F-68 sheets platform for the growth and adhesion of ASCs that exhibits excellent properties for these processes was prepared. *In vivo* study with GO suspended in water on Fisher 344 rats via i.p. administration showed no toxicity up to 100 µg/mL, in spite of part of these GOs accumulated in the intraperitoneal cavity at the first hours. Then, taking together, the data suggest the great potential of graphene oxide sheets as platform to ACSs, as well as, new material for treatment several urological diseases.

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