Cytotoxicity and genotoxicity of graphene oxide scaffold: Human adipose tissue derived mesenchymal stromal/stem cells (AT-MSCs) culture

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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) exhibited excellent properties also for these processes. *In vivo* study with GO suspended in water (100 µg mL^{-1}) on Fisher 344 rats via i.p. administration showed low toxicity. In was also prepared three different scaffolds, such as, graphene oxide/poly(ε-caprolactone) (GO/PCL), graphene oxide/Pegylated amino derivative (GO/PEG-NH_2) and graphene oxide/Pegylated amino derivative/Poly(ε-caprolactone)(GO/PEG-NH_2/PCL) for the growth and adhesion of MSCs and they exhibited excellent MSCs adhesion properties.

In vivo study with GO suspended in water on Fisher 344 rats via i.p. administration showed no toxicity up to 100 µg μL^{-1}, in spite of part of these GOs accumulated in the intraperitoneal cavity at the first hours. However, with these scaffolds no deposition occurred. Then, taking together, the data suggest the great potential of GO scaffolds to MSCs, as well as, new material for treatment several urological diseases. The application of theses GO scaffold is actually in progress since urethral stricture and hypospadias are treatment restricted, since, substitution of the unhealthy urethra with tissue from other origins (bladder, skin or buccal mucosa) are limited.

Keywords: Graphene oxide, cytotoxicity, genotoxicity, stem cells

Classification numbers: 2.00, 2.04, 3.02

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 stricture 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. 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, 2]. A colloidal suspension with small sized GO nanosheets were also prepared *in situ* and exhibited a good biocompatibility of lower cytotoxicity and higher cellular uptake [3,4].

It was reported that the gel formation was long lasting and stable and did not show any severe chronic inflammatory response (GO-poly(lactide-co-glicolide hybride)). Besides this, it was found that adipose-

derived stem cells (ASCs), showed increased adhesion when grown on GO films [5, 6]. Similar structure enhanced the hydrophilic performance, and protein-/inducer-adsorption ability of the nanofibers and GO accelerated also the hMSCs adhesion and proliferation versus pure PLGA nanofiber inducing the osteogenic differentiation [7]. GO nanoflakes were incorporated into a gelatin– hydroxyapatite (GHA) matrix through a freeze-drying technique. Further, GO-GHA induced osteogenic differentiation of human adipose derivatives MSC (hADNSC) without providing supplements like dexamethasone, L-ascorbic acid and β-glycerophosphate in the medium. This differentiation was like those obtained with osteogenic supplements [8].

However, there is still some controversy in terms of cell adhesion morphology when 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. Some important reviews on toxicology of GO discussed these type of problems [9-14].

On view of this facts, in this research was used a representative GO from industrial source (GO-single layer) that it was exhaustively characterized before stem cells cultures. The aim of this work was to prepare GO scaffolds and to study their behavior as a potential nanomaterial for supporting stem cells for use in urologic surgery, with the purpose of reducing infections, regeneration times and even graft rejection during reconstitution in patients with urethral stricture.

2. MATERIAL AND METHODS

2.1. Materials

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. GO was characterized through X-ray diffraction pattern (XRD), X-ray Photoelectron Spectroscopy (XPS), *Nuclear magnetic resonance* (NMR), Raman spectroscopy, Fourier-transformed infrared (FTIR), UV-Vis spectrophotometry, *Thermogravimetric analysis* (TGA), Differential scanning calorimetry (DSC), Transmission electron microscopy (TEM),

Energy Dispersive X-ray Spectrometry (EDS), Dynamic light scattering (DLS) and Nanoparticle Tracking Analysis (NTA) techniques.

Poly (Ɛ-caprolactone) was purchased from Sigma Aldrich, USA (M_w=65,000 g mol^{-1}). 6ARM PEG amine (dipentaerythritol) HCl) was obtained from Jenkem Technology USA (PEG-NH_2). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC) was purchased from Sigma Aldrich, USA.

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 [15]. Graphene oxide paper was made by filtration of the resulting colloid through an filter membrane, Durapore®PVDF(polyvinylidene fluoride) (pore sized 0.22 um, diam 47 mm), followed by air drying and peeling from the filter [16].

2.3. In vivo rats' toxicity study

2.3.1.

To verify the response of the animals' inflammatory system to the presence of GO in solution applied locally (peritoneal cavity) and know its distribution, 0.3 mL of GO solutions (0.01 µg/µL; 0.1 µg/µL and 1.0 µg/µL) were applied via intraperitoneal (i.p.) in a single weekly dose (30 days). The euthanasia of the animals was performed through deep analgesia using 0.3 mL of xylazine and ketamine (1:1) to collect the animals' blood via (i.p.) (Ethics Committee for the Use of Animals approved the study protocol (CEUA/UNICAMP protocol numbers: 3/708-1/2016).

2.3.2. Toxicological and biochemical analyses: It was used 20 rat (Fischer 344) and divided in four groups (n = 5 animals/group). The treatment will be 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) [16].

2.3.3. 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 [17].

2.4. Adipose-derived stem cells (ADMSCs)

ADMSCs from Hemocenter (UNICAMP) were used [18]. The extraction protocol of mesenchymal cells and cellular cultures was based on Choudhery et al. [19]. 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% foetal 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. Synthesis of graphene oxide (GO)/6ARM PEG amine (dipentaerythrito)(PEG-NH_2)

The GO/PEG-NH_2 was prepared using 10 mg of GO dispersed in 18 mL of deionized water. This dispersion was stirred till homogenization in a ultrasound bath for 30 min. To this dispersion 10 mg of EDC ((1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) previously dispersed in 1.0 mL of water and maintaining in the same bath for another 30 min. To this dispersion was added 10 mg of PEG-NH_2 (6ARM PEG amine (dipentaerythritol) HCl) previously dispersed in 1.0 mL of deionized water. This reactional media was maintained under stirring for 24 h in the dark. After this, the mixture was centrifuged at 14.000 rpm for 20 min. The final product was re-suspended in deionized water and centrifuged at the same conditions described before for three times, eliminating the unreacted GO.

2.6. Preparation of scaffold GO/PEG-NH_2 /PCL

Two scaffolds were prepared with Poly(Ɛ-caprolactone)(PCL): GO/PCL and GO /PEG-NH_2/PCL, following the methodology described by Fortunati et al. [20] and Leal et al. [21], to verify the GO dispersion in the polymer, its flexibility and toughness as well as improved adhesion to tissue (e.g., bladder).

First, 1.5 g of PCL were dissolved in 20 ml of chloroform (Merck, Germany) and kept under stirring for 2 hours for complete dissolution of the polymer. At the same time 0.5 mg of GO were added to 10 ml of chloroform and placed in ultrasonic bath Unique, Model USC 700 for 30 min to disperse GO. This step was reduced to 5 min when used GO/PEG-NH_2, showing the significant improvement in dispersibility of the new conjugate GO.

Then, the dispersed solution of GO was dissolved aggregate the PCL solution and placed on a magnetic stirrer for homogenization for 10 minutes. The pre-test concentrations to define the optimum ratio were applied previously by Lee et al. [22] and Kim et al. [23]. The final solution obtained was poured into glass mold (glass vessel 10 x15 cm) to complete evaporation of the solvent to room temperature without controlled atmosphere to obtain the scaffolds.

2.7. Growing stem cells in the presence of conjugate GO

Inoculums of standards mesenchymal stem cells were 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 h to achieve maximum log growth for the time of incubation in an incubator at 37°C in 5% CO_2 with 10% calf serum [24]. 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°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 37ºC. 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.

2.8. Characterization

2.8.1.Scanning electron microscopy (SEM)

A FEI QUANTA FEG250 SEM instrument, operating at 5 kV was used. The sample were deposited and coated with a conducting layer of Iridium that gives a fine grained coating and is easily applied in a sputter coater (high vacuum sputter Baltec MED 020).

2.8.2. X-ray diffraction (XRD) patterns

X-ray diffraction (XRD) measurements were carried out on an X-ray diffractometer (Shimadzu XRD 7000). The patterns with Cu K_α radiation ($\lambda = 0.15406$ nm) at a 40 kV voltage, 30 mA current with a 2° min^{-1} scanning rate were recorded in the 5°- 90° 2θ region.

2.8.3. Cellular viability by fluorescence microscopy

The LIVE / DEAD® technique was performed to check the cell viability. For this, it was used the BaclightTM kit (Molecular Probes) consisting of a mixture with two markers: green fluorescent marker SYTO® 9 (480/500 nm excitation/emission) and a red fluorescent label propidium iodide (490/638 nm excitation/emission). The SYTO 9 mark all bacteria present in the sample, viable and non-viable. Conversely, propidium iodide penetrates only cells with damaged cell membrane (nonviable), causing reduction of SYTO 9 when both dyes are present. Thus, when there is an appropriate mixture of the two markers, the cells that have intact cell membrane (viable) are marked in green fluorescence, while cells that exhibit damage in the cell membrane (non-viable) are marked in red fluorescent [25]. After the incubation period, all the wells were washed with PBS twice to remove cells not adhered to the scaffolds and the images were acquired in a microplate reader Cytation 5 (Biotek, Vermont, USA) using the GFP

filter (469/525 nm excitation/emission) for reading the Calcein-AM and RFP (531/593 nm excitaion/emission) for reading the ethidium homodimer.

2.9. Cytotoxicity through histochemistry of the target organs

To this study it was analyzed the inflammatory system behavior of the animals by the GO presence applied locally (peritoneal cavity) after administration of 0.3 mL of GO solutions (0.01 /µL; 0 , 1 µg/µL and 1.0 µg/µL) intraperitoneally (ip) in a single weekly dose (30 days). Euthanasia of the animals was performed by deep anesthesia with use of 0.3 mL of xylazine and ketamine (1: 1) to perform blood collection the animals (i.p.). The collecting some target organs such as the peritoneum, liver and spleen were performed to observe the penetration range and the local response of each of these organs.

After collecting the bodies, they were placed directly in Bouin fixative and left for 12 hours. After this period the pieces have undergone 10 rinses in 70% alcohol. For the inclusion of this material parts have undergone different processing steps. Drying: The samples were placed in 80% alcohol, 100% alcohol I and II, 100% alcohol III and IV for 30 min in this order. Diafanization: The pieces were placed in alcohol/xylene for 15 min and xylene I and II and xylene/paraffin (2: 1) for 30 min. Inclusion: The pieces were placed in paraffin wax I and II for 1 h, then to be positioned inside the mold and covered with paraffin, cool down and form blocks. Histological sections (5 µm) were prepared using Micrometer (Rotary Microtome Slee CUT5062 RM 2165 (Slee Mainz, Mainz, Germany). The slides were stained by hematoxylin-eosin (HE). Before the coloration process, the slides were placed in an incubator (60°C) between 30 min and 1 h prior to deparaffinization. After this step the blades passed the hydration process: Xylene I - 10 min, Xylene II - 10 min, Xylene/Alcohol - 5 min, absolute alcohol - 5 min, alcohol 80% - 5 min, alcohol 70% - 5 min and water - 5 min. Complete hydration slides were placed in Harris Hematoxylin stain for 30 s and after were left in water for 10 min. The slides were placed in Eosin Alcohol dye for 6 s and then dehydration

was performed: Alcohol 80% - 5 min Alcohol 95% - 5 min Alcohol 95% - 5 min Absolute alcohol I - 5 min Absolute alcohol II - 5 min Xylene I - II 5 min and Xylene - 5 min. With the completion of this stage the assembly of the blades was performed. In each sample was added one drop of Entellan® (Merk) on the cutting and the coverslip was positioned to complete the assembly of the blades, which were for 2 days at room temperature to dry and are ready for viewing under the microscope.

Through microscopy, signs of inflammation, hemosiderin occurrence, and GO deposition were the main changes analyzed and photographed after performing this technique.

2.10. Genotoxicity – Micronuclei counts

To 0.3 mL of GO suspension were applied (0.01 µg/µL, 0.1 µg/µL and 1.0 µg/µL) intraperitoneally (ip) in a single dose weekly and after 30 days the animals were euthanized with 0.3 ml xylazine anesthetic/kuetamina (1: 1) (ip), and the right femurs were removed and processed with saline and collecting the medulla washing as described below.

The results when compared to controls, negative (healthy animals) and positive (animals induced with a single dose of MNU (N-methyl-N-nitrosourea)) showed the expected results: the higher the concentration of GO, the greater the damage and therefore genotoxicity (Table 1). Still were observed at all concentrations, GO clusters of red cells, indicating the migration of the compound of the peritoneum (application site) to the bloodstream.

For the frequency count of micronuclei in 3000 erythrocytes of each animal (Fisher 344 rats), blood smear slides were used collected from the bone marrow of an animal femur and prepared on the day of the sacrifice of animals. For this, after sacrifice and exposure of the femur and vise fixation was performed at breaking the distal epiphysis, puncture with physiological serum (PBS) at room temperature with 0.8 needle, performing repeatedly (3 times) washing of the femur, collecting all liquid . The collected material was placed in microtube added volume of

0.9% PBS to complete 1 mL and centrifuged for 5 min at 1000 rpm. Then, it was removed the supernatant until a 1: 1 ratio with the deposit, homogenized and made the smear slide. After drying (few min) they were fixed in methanol for 10 min and stained with Giemsa modified in deionized water (1:20) for 15 min. After the complete drying of the blade was added Permount thin layer (glue) and cover slip. Images were obtained by optical microscopy immersion lens (100x). It was carried out the counting of micronuclei in animals in the acute phase of the test (24 h after a single dose application intraperitoneally) and after chronic phase of the trial (30 days with weekly i.p. applications).

2.11. Applicability of scaffolds

A practical test with the GO sheets and from the GO *scaffold* was carried out using all the elements for surgery to know the flexibility and resistance of these materials at the moment of surgical uses.

Since, the nest step, it should be to exert a suture using the GO sheets, the pressure required to lock the point damaged the scaffold, causing cracks which eliminated use in surgery. The solution was to set another material that improve the performance of this scaffold and a new faster approach and let the surface more uniform on the scaffold. So new formulations and procedure for obtaining the membrane were studied and performed.

It was conducted a survey of characteristics of different polymers that could modify the GO increasing the flexibility of the scaffold, preventing the GO hardness feature to accomplish the suture tissue. In this search mainly it was chosen the six-membered branched PEG (PEG 6ARM amine (dipentaerythritol) HCl, with an end of each member consisting of a primary amine (R-NH2) with a molecular weight of 15 g/mol, and others, such as Pluronic and Polycaprolactone. This polymer as it has known characteristics such as biocompatibility, favoring protein binding to structures, modulator of cellular interactions and favoring tissue [26].

3. RESULTS AND DISCUSSION

3.1. Characterization of GO commercial sample

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 (some examples of the analyses, Fig.1).

3.2. Toxicity of GO in vivo

Previously, it was shown that *in vitro* and *in vivo* GO did not exert any toxic effect up to 100 µg/µL and besides this no hemolysis was also found [24]. *In vivo* study with GO suspended in water on Fischer 344 rats via intraperitoneal administration showed low toxicity, since part of them they accumulate in the intraperitoneal cavity. Similar results with GO on bald/c mice preliminary results were reported previously [27].

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).

In vivo study with GO suspended (1.0 µg/µL) in water on Fischer 344 rats via intraperitoneal administration showed low toxicity, since part of them they accumulate in the intraperitoneal cavity. Similar results with GO on bald/c mice were reported previously [28, 29].

This probably did not affect the final absorption of GO via intraperitoneal administration, since in another experiment with this GO (100 µg/µL) 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 [30].

Images in optical microscope (Olympus BX51 fluorescence microscope) of MSCs cell viability in the presence of GO was studied (Fig. 2). In all concentrations was observed cell growth, even in a different

intensity and with slight morphological changes. The same results were observed after 72 h with small variations. Also, it was observed the migration of MSCs toward the edge of the wells at concentrations of 1-50 µg/µL after 24 h (not shown).

Only the solution with a concentration of 1 µg/µL cells behaved more like the control, as to its growth as its morphology. The decrease was significant cell concentration from 5 µg/µL GO, including being difficult to find viable cells at the time of making the images. The results of scanned images, the concentration of GO above 5 µg/µL inhibits or capture stem cells that are not adhered and therefore end up being removed from wells at the time of washing.

These results agree with results published previously [22-24, 31-33], describing the toxicity exerted by GO on cell growth of MSCs, indicating that the use of concentrations of GO suspension above 1 µg/µL to be thoroughly evaluated. It could be seen in these tests that happens capture of the cells by GO, since the number of available cells as observed by the significant fluorescence decreases as the concentration increases, and probably at the time of washing the wells all the cells attached to the GO are discarded.

3.3. Characterization of conjugate of GO/PEG-NH_2 and GO/PEG-NH_2/PCL by SEM

After the synthesis of conjugate of GO with PEG-NH_2 and of GO/PEG-NH_2/PCL as previously described both conjugates were characterized by SEM.

Images of SEM (Fig.3) shows clearly between fine sheets of GO, that exhibits a typical transparence and little agglomeration of these structures (Fig. 3A); differently from GO-PEG-NH_2 powder, which already has an opaque appearance and the layers are not more dispersed, with appearance of dry leaves of trees (Fig. 3B-D).

3.4. Characterization of conjugate of GO/PEG-NH_2 and GO/PEG-NH_2/PCL by XRD.

In the XRD of the main diffractions of the powders of GO and GO/PEG-NH_2 can clearly observe the diffraction peak of pure GO (hydrated) (Fig.4A) is located at 11.5° 2Θ

corresponding to GO interlayer distance of approximately 0.8 nm, which it is an important indicator of intercalation water-GO. In the presence of PEG-NH_2 the peak at 11.5º is shifted to 6º 2Θ (Fig. 4B), which corresponds to a basal spacing of approximately 1.4 nm, due to the incorporation of PEG-NH_2 molecules that has 6 arms with angled structures capable of distancing layers GO increasing the interlayer spaces. This it was observed previously by Zhang et al. [34] in a report where the authors used PEG 20000, where an increase in interlayer spacing gradually increase according to increase of PEG concentration.

Together with these data investigation of the change physicochemical properties, such as in the rheological behavior because of Graphene nanoparticles, showed that viscosity of the composites was measured as well, the photo-thermal energy conversion experiment indicates that the PEG/Graphene nanoparticles composites show better performance in photothermal energy conversion [35].

In the Figure 4C shows the XRD patter of PLC membrane and in the Figure 4D is shown the scaffold (GO/PEG-NH_2/PCL) in which does not appear any pattern related to GO, since the PCl concentration was elevated related to GO concentration.

3.5. SEM microscopy of PCL membrane and scaffold (GO/PEG/PCL)

Through the images of the obtained scaffolds (GO-PCL and GO/ PEG-NH_2/PCL) it was checked the surface characteristics of both sides of the scaffolds (side in contact with the forming surface (glass) and side contact through evaporation environment (oxygen atmosphere). Side B (bright) was standardized as the side facing the support surface at the time of evaporation. M side (mat) as the side facing the environment (Fig. 5), especially in regard to porosity (presence of micro holes) due to the solvent evaporation speed used to dilute the GO. From this analysis it was chosen the B side to inoculate MSCs

in the scaffolds during the cell viability test, because it was desired to check this adhesion and growth of MSCs without physical properties of the formed scaffold that facilitates this adhesion.

3.6. Cellular viability of MSCs in the scaffold (GO/PEG/PCL)

Standard inoculum of MSCs were obtained from human adipose tissue s previously described and the LIVE/DEAD technique was used to verify the cellular viability.

During the 24 h period (figure no exhibited, see preliminary outcomes in an extended abstract ref. [36]) set by the preliminary tests, the cells have no morphological changes and find fields with a greater number of cells, indicating favoring growth in the presence of the scaffold, showing evidence of synergistic interaction of the scaffold and MSCs, these results agree with results shown in a previous reported. It was discussed that like GO substrates, have been already demonstrated to stimulate human MSCs to differentiate into adipocytes, to induce the differentiation of neural stem cells (NSCs) into neurons in three dimensional (3D) porous structure and of induced pluripotent stem cells into the endodermal lineage [37].

3.7. Cytotoxicity through histochemistry of the target organs

The analysis of target organs by histochemistry shows a dose dependent effect. Figure 6. shows regions with moderate inflammation and hemosiderin signals in the group treated with GO suspension at 0.01 µg/µL concentration (Fig.6 f, g). At 0.1 µg/µL GO suspension a large region of intense inflammation, large regions with hemosiderin and certain regions with GO deposition (Fig.6 h-o).

Histopathological analyses of spleen and liver showed regions with intense inflammation, hemosiderin occurrence and large GO deposition after a 1.0 µg/µL of GO suspension during 30 days.

In the case of GO-PCL and GO/ PEG-NH_2/PCL only small effects on organs were observed on the animals by histopathological analyses.

3.8. Genotoxicity: Micronuclei counts

The results when compared to controls, negative (healthy animals) and positive (animals induced with a single dose of MNU (N-methyl-N-nitrosourea)) showed the expected results: the higher the concentration of GO, the greater the damage and therefore genotoxicity (Table 2).

Still were observed at all concentrations, GO clusters of red cells, indicating the migration of the compound of the peritoneum (application site) to the bloodstream (results not shown). At the suspension of 0.01 µg/µL GO was observed no effect with a similar value that the negative control. GO-PCL and GO/ PEG-NH_2/PCL exhibited very low effect on micronuclei (results not shown)

3.9. Applicability of the scaffolds for use as support of MSCs in urethral stricture.

In future terms in the applicability of scaffolds, in preliminary tissue suture tests the GO was not flexible enough, causing breaks in the GO sheets. The solution was to set another material that improve the performance of this scaffold and a new faster approach and let the surface more uniform on the scaffold. So new formulations and procedure for obtaining the membrane were studied and performed.

It was conducted a survey of characteristics of different polymers that could modify the GO increasing the flexibility of the scaffold, preventing the GO hardness feature to accomplish the suture tissue (Pluronic, Polycaprolactone-PCL). The best result was with the six-membered branched PEG (PEG 6ARM amine (dipentaerythritol) HCl, with an end of each member consisting of a primary amine (R-NH2) with a molecular weight of 15 g/mol associated to PCL. This polymer as it has known characteristics such as biocompatibility, favoring protein binding

to structures, modulator of cellular interactions and favoring tissue regeneration [26]. Then, this GO-PEG-NH_2 that was biocompatible and low toxicity it will be used in the next steps for animals treatment with urethral wound and stricture.

4. CONCLUSIONS

GO in water suspension exhibited an inhibition of the cell growth over 5 µg μL^{-1}. The prepared GO/PCL, GO/PEG-NH_2 and GO/PEG-NH_2/PCL scaffolds for the growth and adhesion of MSCs exhibit excellent properties. *In vivo* study with GO suspended in water on Fisher 344 rats via i.p. administration showed no toxicity up to 100 µg/µL, despite part of these GOs accumulated in the intraperitoneal cavity at the first hours. However, this study with these new scaffolds did not present this deposition. Then, taking together, the data suggest the great potential of GO scaffolds to MSCs, as well as, new material for treatment several urological diseases. The application of theses GO scaffold is in progress since urethral stricture and hypospadias are treatment restricted, since, substitution of the unhealthy urethra with tissue from other origins (bladder, skin or buccal mucosa) are limited.

Author contributions

ND, ACML WJF, MD, HJC- Conceptualization, Methodology, Writing-original draft, Review and Editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

All animal procedures were in fulfillment with the Ethical Principles in Animal Research, validated by the National Institutes of Health Animal Research Guidelines and ARRIVE guidelines. The University of Campinas (UNICAMP, Brazil), Ethics Committee for the Use of Animals approved the study protocol (CEUA/UNICAMP protocol numbers: 3/708-1/2016.

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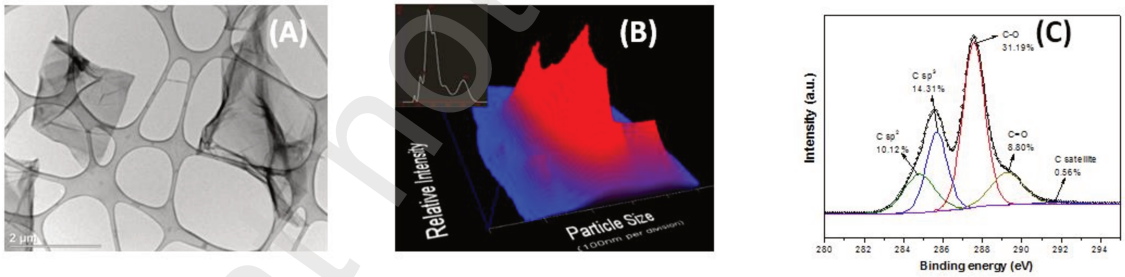

Figure 1. (A) TEM-MSC 2100 – JEOL showing transparent sheets and slightly wrinkly (~1 nm thickness); (B) NTA NanoSight LM-10, average size of 355 nm and PI 0.38; (C) K-Alpha X-ray Photoelectron Spectrometer (XPS) – Thermo (C_{1S} showed a C/O ratio of 1.7 and a C_{sp2}/C_{sp3} ratio of 0.7).

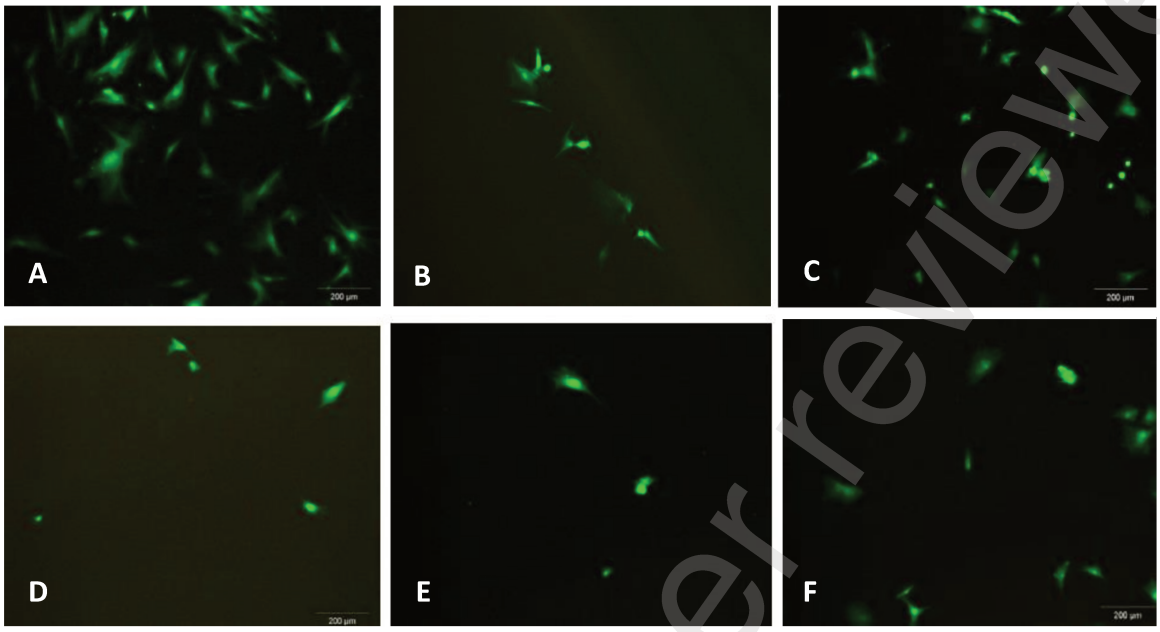

Figure 2. Fluorescence micrographs of steam cells. Images of MSCs viability (5X10^3 cells/mL) in GO suspension after 24 h of incubation. A: MSCS control. B: in the presence of 1.0 µg/µL GO. C: in the presence of 5 µg/µL GO. D: 10 µg/µL GO. E: 25 µg/µL GO. F: 50 µg/µL GO.

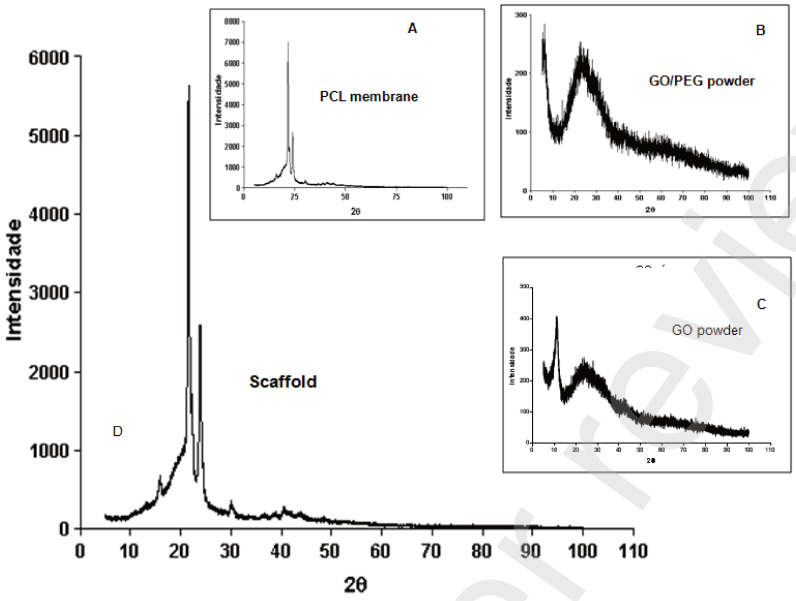

Figure 4. XRD pattern showing the shift picks of GO powder when incorporated PEG-NH2 to the GO/PEG powder and PCL membrane versus GO/PEG/PCL *scaffold.*,.

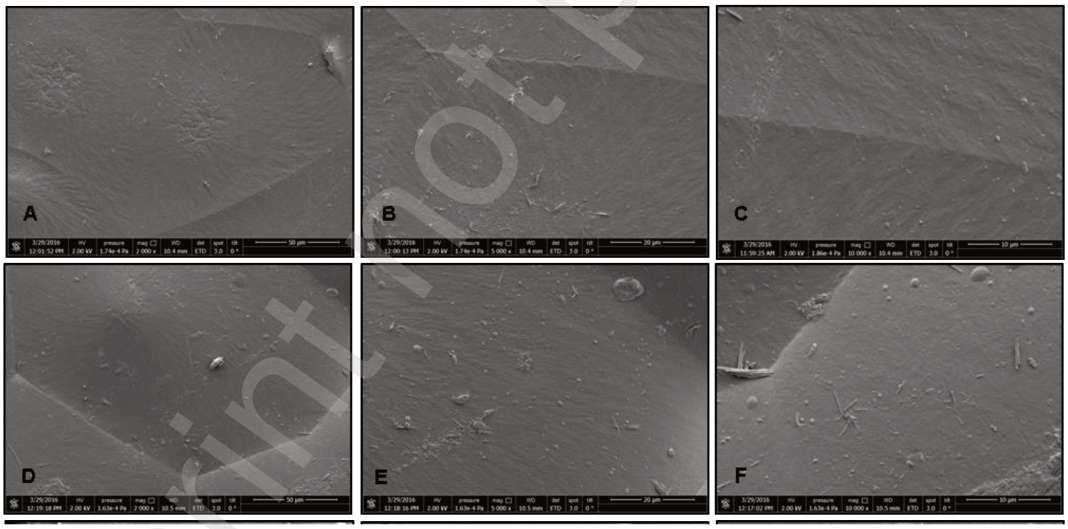

Figure 5. SEM images of scaffold of PCL. Side B do scaffold: A (scale 50 µm). B (scale 20 µm). C (scale 10 µm). Side M of scaffold D (scale 50 µm). E (scale 20 µm). F (scale 10 µm).
As verified through the obtained images, differences between the two sides (B and M),

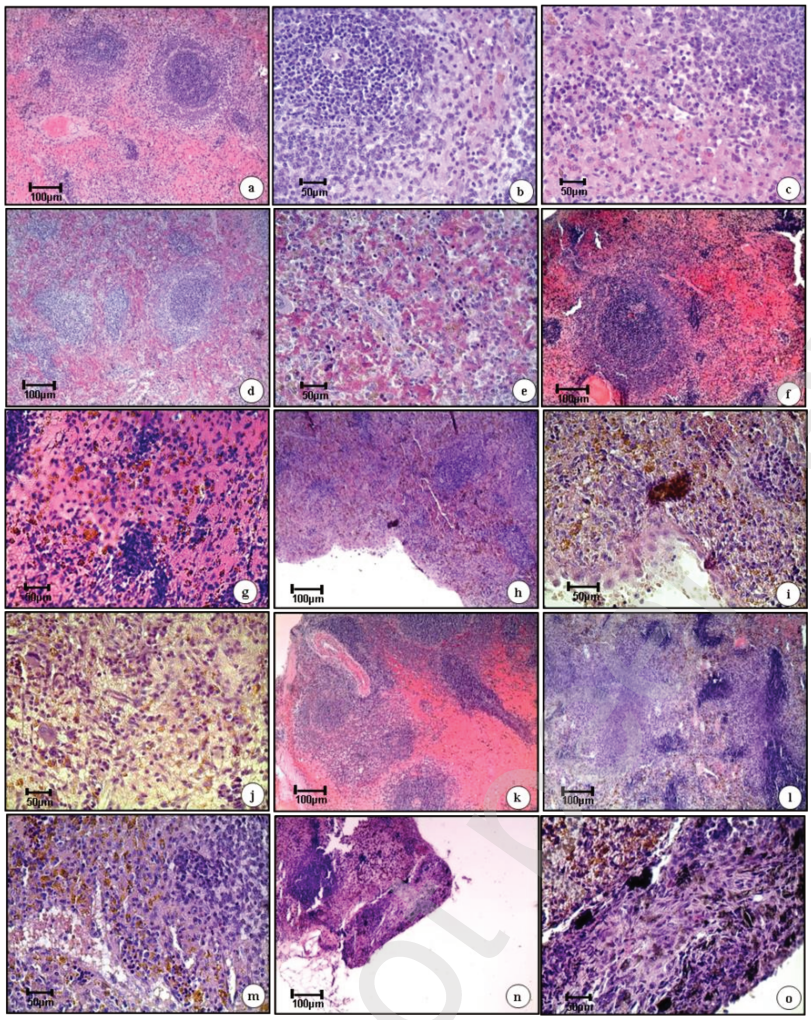

Figure 6. Images of spleen of the treated animals with GO suspensions at different concentrations for 30 days with weekly single dose i.p. a, b, c, d and e show preserved tissue of the control group (normal spleen). Images f and g show regions with moderate inflammation and hemosiderin signals in the group treated with 0.01 µg/µL GO suspension. Images h, i, j, k, l, m, n, o show large regions of intense inflammation, large regions with hemosiderin and certain regions with GO deposition in the group treated with 0.1 µg/µL GO suspension.

Table 1: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine values showing biochemical parameters of hepatic and renal functions after 24 h of i.p. administration of GO (10 µg/µL) and GO (100 µg/µL).

Samples	ALT ($U\ L^{-1}$)	AST ($U\ L^{-1}$)	Urea ($mg\ dL^{-1}$)	Creatinine ($mg\ dL^{-1}$)	*P value*
Control	28.7±2.1	51.7±2.1	29.7±1.5	0.27±0.06	$P < 0.01$
GO (10 µg/µL)	31.0±2.8	53.5±2.1	31.5±0.7	0.31±0.06	$P < 0.01$
GO (100 µg/µL)	32.5±3.5	53.0±2.8	27.0±2.8	0.26±0.03	$P < 0.01$

Data expressed as average ± standard deviation (n= 03). All the measurements indicated no differences between them by the Turkey test.

Table 2. Micronuclei counts from blood of the animal GO treated with different concentrations via i.p administration

Animals	Group	Red blood cells	MicronucleI	Average
WF39/15	Negative control	3000	3	10
WF218/15	Positive control	3000	283	283
WF44/15	1.0 µg/mL	3000	41	-
WF46/15	1.0 µg/mL	3000	57	-
WF47/15	1.0 µg/mL	3000	26	-
WF48/15	1.0 µg/mL	3000	10	34
WF49/15	0.1 µg/mL	3000	35	-
WF50/15	0.1 µg/mL	3000	16	26
WF54/15	0.01 µg/mL	3000	17	-
WF55/15	0.01 µg/mL	3000	9	-
WF56/15	0.01 µg/mL	3000	9	-
WF57/15	0.01 µg/mL	3000	4	10