Polymeric film of 6-arm-poly(ethylene glycol) amine graphene oxide with poly (ε-caprolactone): Adherence and growth of adipose derived mesenchymal stromal cells culture on rat bladder

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Abstract. Nanotechnology has been more present in different fields related to health. 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 adipose-derived stem cells. Advanced techniques for the growth, differentiation and proliferation of adipose-derived stem cells were used, as well as the characterization of graphene oxide sheets. For this study, it was prepared the graphene oxide/6 ARM-Poly(ethylene glycol) amine films with poly (ε-caprolactone). The graphene suspension in organic solvent was prepared by using an ultrasonicator bath and subsequently, the film was formed by solvent evaporation. Total characterization of graphene oxide/6 ARM-Poly(ethylene glycol) amine films with poly (ε-caprolactone). The graphene suspension in organic solvent was prepared by using an ultrasonicator bath and subsequently, the film was formed by solvent evaporation. Total characterization of graphene oxide/6 ARM-Poly(ethylene glycol) amine films with poly (ε-caprolactone) film was carried out. It was tested growth and adhesion of adipose-derived stem cells on the film, as well as, were verified the histopathological effects of this scaffold when implanted in the urinary bladder to repair the lesion. Our results demonstrated that this scaffold with adipose-derived stem cells enhanced the repair in rat urinary bladder defect model, resulting in a regular bladder. Improved organized muscle bundles and urothelial layer were observed in animals treated with this scaffold with adipose-derived stem cells compared with those treated only suture thread or scaffold. Thus, our biomaterial could be suitable for tissue engineered urinary tract reconstruction.

1. Introduction
Within the symptomatology that affects urologic patients; voiding dysfunction is one of the major problems with direct impact on quality of life. These symptoms can be directly related to urethral stricture, which occurs in the urethra. Urethral stricture can result from inflammatory, ischemic or...
traumatic reactions [1]. These processes lead to the formation of scar tissue; this decreases the calibre of the urethral lumen, causing resistance to antegrade flow of urine. Treatment reports of urethral stenosis date back to the Egyptians 4000 years ago and very little have changed until the middle of last century. Treatment today is still a challenge, which directed many researchers from different centres, to study not only the use of stromal mesenchymal cells (SMCs) for reconstitution of urological tissue by promoting healing acceleration, as well as demonstrate the possibility of differentiation of these cells stromal mesenchymal cells in particular applied for each region [2, 3]. Studies have demonstrated the differentiation of these in urothelial cell lines [4]. In 2012 a survey conducted by Frennette et al. [2] showed a number of published papers related to stromal mesenchymal cells were divided into 4 groups: immunomodulation, tissue protection, regenerative medicine and improvement grafts. Bioactive molecules secreted by stromal mesenchymal cells exert trophic effects on the surrounding tissue, causing: (a) anti-apoptotic effects on cell tissue specific, (b) immunomodulatory effects on cells of the immune system, (c) increasing angiogenesis, and (d) chemotaxis to other young cells committed to the repair process [5]. Thus, reducing the initial inflammatory response may occur in some ischemic diseases, restoration of blood supply and adequate tissue repair, depending on the extent and severity of tissue damage. Therefore, the last two years extensive research in the area of nanotechnology presents and pointed out the possibility of having a biocompatible support using graphene oxide (GO) in the construction of scaffolds that allows the growth and stromal mesenchymal cells differentiation [6-13]. One of these studies examined the interference of the graphene oxide and reduced graphene oxide (R) on the differentiation of skeletal myoblasts and morphology of mice. The authors found that graphene oxide and its reduced form did not affect negatively this differentiation, but acted as stimulators of the same, without change morphologically these cells. The graphene oxide was able to increase in the viability 1.53 times in two days and 1.24 fold after 4 days, compared to control cells, is better than the reduced graphene oxide [12]. But to develop studies with these materials should be considered the risks to animal and human health, and therefore should be studied toxicological tests for minimize risk before moving in the medical area [14, 15]. These studies can be said the promising future of graphene oxide as material to be used in the medical field.

2. Methods

2.1. Conjugate synthesis GO/6 ARM-PEG-amine

In order to obtain the conjugate GO/6 ARM-PEG-amine, 10 mg of graphene oxide (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) used which was dispersed in 18 mL deionized water. The stirred dispersion to homogenization and held in an ultrasound bath for 30 min. Then added 10 mg of EDC (hydrochloride 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) previously dispersed in 1.0 ml of water and kept at ultrasonic dispersion bath for 30 min with subsequent agitation for mis 30 min. Thereafter, was added 10 mg of PEG (6 ARM-Poly (ethylene glycol) amine (6 ARM-PEG-amine) (15kD) previously dispersed in 1.0 ml of deionized water. The reaction medium was maintained under stirring for a period of 24 h in the absence of light.

The next day, the mixture was centrifuged at 14,000 rpm for 20 minutes. Thus, the conjugate was resuspended in deionized water and centrifuged under the same conditions described above. This procedure was repeated 3 times to remove unreacted 6 ARM-PEG-amine with graphene oxide. Preliminary studies have shown that it is possible to obtain stable suspensions of graphene oxide in deionized water (1mg / ml) for up to 10 days. The suspensions of graphene oxide (1.0 mg/mL) will be prepared in sterile deionized water and conditioned in 15 mL Falcon tubes (sterile) when placed in ultrasonic bath for 30 minutes.
2.2. Obtaining the scaffold GO/6 ARM-PEG-amine/PCL
First, 1.5 g of poly (ε-caprolactone) (PCL) (80 kD) were dissolved in 20 mL of chloroform (Merck, Germany) and kept under stirring for 2 h for complete dissolution of the polymer. At the same time 0.5 mg of graphene oxide were added to 10 mL of chloroform and placed in ultrasonic bath Unique, Model USC 700 for 30 min to disperse graphene oxide. This step was reduced to 5 min when used GO/6 ARM-PEG-amine, showing the significant improvement in dispersibility of the new graphene oxide conjugate. Then dispersed solution of graphene oxide was dissolved and aggregated to the PCL solution and placed on a magnetic stirrer for homogenization for 10 min. The pre-test concentrations to define the optimum ratio were applied by Lee et al. [16] and Kim et al. [13]. The final solution obtained was poured into glass mold (glass vessel 10 cm X 15 cm) to complete evaporation of the solvent to room temperature without controlled atmosphere to obtain the scaffolds.

2.3. Characterization of the conjugate GO/6 ARM-PEG-amine/PCL scaffold
2.3.1. Scanning Electron Microscopy (SEM) of the membrane scaffold PCL and GO/6 ARM-PEG-amine/PCL
As done with samples of graphene oxide and graphene oxide conjugate, the morphologies of scaffolds GO/6 ARM-PEG-amine/PCL were studied in the scanning electron microscope for issuing FEI QUANTA FEG250 field, operated at 5 kV. Samples scaffolds were placed under the adhesive tape and the covered sample port with a thin layer Iridium, using a high vacuum sputter modular Baltec O20 MED.

2.3.2. X-Ray Diffraction (XRD) GO/6 ARM-PEG-amine, PCL membrane and scaffold GO/6 ARM-PEG-amine/PCL
The comparative analysis was made to be able to compare the peaks of different compounds and verify that there was indeed an interaction between them, using XRD equipment by Philips X’Perta.

2.4. Obtaining adipose mesenchymal stromal cells (AMSCs)
The AMSCs will be obtained from human adipose tissue undergoing liposuction surgery under general anesthesia. All procedures were approved by the Ethics Committee of the UNICAMP (process CEP-UNICAMP- No. 839/2008). The Term of Free and Informed Consent (process CEP-UNICAMP- No. 839/2008) was applied to all patients undergoing liposuction which have agreed to the donation. The selected patients were between 25 and 50 years and were excluded diabetic and hypertensive patients. All the procedures performed involving adipose mesenchymal stromal cells as extracting mesenchymal stromal cells and cell culture; immunophenotype of the mesenchymal stromal cells by flow cytometry; mesodermal differentiation in vitro; chondrogenic differentiation; osteogenic differentiation; adipogenic differentiation; cytochemical analysis of differentiated mesenchymal stem cells; Confocal immunofluorescence microscopy; activity of the enzyme telomerase and cytogenetic were adapted from the techniques used by different authors by the team of Prof. Dr. Angela C.M. Luzo, molecular biology laboratory. - Blood Center - UNICAMP, Campinas [17].

2.5. Viability of cells on the scaffold AMSCs GO/6 ARM-PEG-amine/PCL
Inoculum was prepared from adipose mesenchymal stromal cells laboratory standards obtained from human adipose tissue in DMEM (Dulbeco's Modified Eagle Medium), which were incubated for 72 h to attain the maximum log growth for the time of incubation in an incubator at 37°C in 5% CO2 with 10% bovine serum (mother inoculum) [8].

Thus, from the mother inoculum (initial) was prepared one MSCs solution through detachment of these adherent cells in disposable petri dish (diameter 150 mm) using 1 mL of trypsin (0.005%) for 3 min at 37°C, made washing with PBS (phosphate buffered saline (0.1 M)). Cell viability was verified by trypan blue staining technique and counting in a Neubauer chamber [18]. This cell solution was used to inoculate scaffolds. After placement of the scaffold, aseptically, each well received 125 uL of a solution (6x105 cells / ml) which was added 1.0 ml of DMEM with 100 of 2% bovine serum and and they were inoculated to 37°C for 24 h. The LIVE / DEAD® technique was performed to check the cell viability and the images were acquired in microplate reader Cytation 5 (Biotek, Vermont, USA) using the GFP filter (ex / at ~ 469 nm / ~ 525 nm) for reading Calcein-AM and RFP (ex / at ~ 531 nm / ~ 593 nm ) for reading the ethidium homodimer.
2.6. Obtaining the bladder injury and implantation of the scaffold

A total of 12 rats of the Fischer 344 strain were divided into 4 groups (3 animals per group) and with the animal placed, there were aseptic in the lower third of the region of the abdomen then the animals were anesthetized i.p. using 0.2 mL of xylazine / ketamine (1: 1) and the incision performed. Experimental group (n = 3 animals per group) was divided at Control group, Group suture thread attached with adipose mesenchymal stromal cells, Scaffold Group and Scaffold group adhered to adipose mesenchymal stromal cells which the latter two were also sutured with suture thread from adipose mesenchymal stromal cells. The scaffold was positioned so that the side joined with the adipose mesenchymal stromal cells stay facing the lumen of the organ and the suture was performed. To check if there escape of urine, after suturing performed filling the same using 0.9% saline stained with blue pigment. A urinary catheter (22 gauge) was introduced into the animal's urethra and a small amount of serum was given slowly and observing whether there was leakage or not. After 30 days of follow up, the rats were sacrificed and the bladder was collected using mooring technique where after deep anesthesia procedure and opening of the abdomen, the bladder was emptied completely by introducing catheter and then was completed solution Bouin (fixer) and tied at the bottom portion to be fixed with total volume. The histopathology was made to assess healing and inflammatory processes.

2.7. Histopathological analysis

After fixed with Bouin liquid for 12 h the bladder follow standard fixation procedures, dehydration, and inclusion diafanization using Paraplast Plus (ST. Louis, MO, USA). The materials was sectioned on rotary microtome CUT5062 (SLEE MAINZ, Munich, Germany) with a thickness of 5 microns, stained with hematoxylin and eosin (HE) and Masson, and photographed the light microscope Leica DM2500 (Leica, Munich, Germany) equipped with DFC295 camera (Leica, Munich, Germany) [19]. The following criteria were observed: angiogenesis, urothelial hyperplasia, inflammatory infiltration, foreign body giant cell reaction around the suture and scaffolds and collagenous.

3. Results and Discussion

3.1. Characterization of the scaffold:

3.1.1. Scanning Electron Microscopy (SEM) of the membrane scaffold PCL and GO/6 ARM-PEG-amine/PCL

The images of SEM from GO-PCL and GO/6 ARM-PEG-amine/PCL scaffolds showed the surface characteristics of both sides of the scaffolds: B-side (bright) was defined as the side in contact with the forming surface; F-side (front) was defined as the side in contact through evaporation environment (e.g., oxygen environment contact). Due to the solvent evaporation rate used to dilute the graphene oxide, and the surface appeared more homogenous and soft (Fig. 1, samples A,C,E,G) that the F-side (Fig. 1, samples B,D,F,H), the B-side was chosen to inoculate the adipose mesenchymal stromal cells in the scaffolds at the cell viability test, to facilitate the adhesion and growth of adipose mesenchymal stromal cells.
Figure 1. SEM images of the B-side (A – 100X, C – 2.000X, E – 5.000X, G – 10.000X) and F-side (B– 100X, D – 2.000X, F – 5.000X, H – 10.000X) from GO/6 ARM-PEG-amine/PCL scaffold.
3.1.2. X-Ray Diffraction (XRD) GO/6 ARM-PEG-amine, PCL membrane and scaffold GO/6 ARM-PEG-amine/PCL

The diffraction peak of pure graphene oxide was located at 11.5° (2θ), corresponding to distance of graphene oxide interlayer with approximately 0.8 nm, which is an important indicator of interleaving between graphene oxide sheets, due to its aromatic groups. In the presence of 6 ARM-PEG-amine, 11.5° diffraction peak was shifted to 6°, which corresponds to basal spacing of approximately 1.4 nm, due to incorporation of 6 ARM-PEG-amine (6 arms with angled structures capable of distancing layers graphene oxide, increasing spaces of the interlayer). By comparing the membrane PCL and the scaffold itself, there was no observed diffraction peak of GO in the scaffold, due to the high concentration of the PCL (Fig. 2).
**Figure 2.** DRX showing displacement graphene oxide peaks powder (A) when incorporated into the PEG in GO/ powder 6 ARM-PEG-amine (B), and PCL membrane scaffold (C) versus GO/6 ARM-PEG-amine/PCL (D).

3.2. Viability of cells on the scaffold AMSCs GO/6 ARM-PEG-amine/ PCL

When compared with controls (fig. 3A and B), the incubation after 24 h of adipose mesenchymal stromal cells with GO/6 ARM-PEG-amine/PCL scaffold, in relation to cell viability, there were no morphological changes in adipose mesenchymal stromal cells (Fig 3C and D), but it was possible to detect that the GO/6 ARM-PEG-amine/PCL scaffold increased adipose mesenchymal stromal cells proliferation, indicating synergistic interaction (Fig. 3).
Figure 3. Photomicrographs A and B showing the adipose mesenchymal stromal cells grow control. The photomicrographs C and D show proliferation of the adipose mesenchymal stromal cells in culture incubated with GO/6 ARM-PEG-amine/ PCL scaffold.

3.3. Histological analysis

The histological results from 12 rats of the Fischer 344 strain, showed that angiogenesis process was more intense in the Scaffold+ADMSCs group in relation to Scaffold and Suture thread groups, in which the angiogenic process was predominantly moderate (Fig.4). In contrast, inflammatory process and giant cell reaction were numerically greater in the Suture thread in relation to Scaffold and Scaffold+ADMScs groups, which showed moderate tissue reaction (Fig. 4). The collagenization was intense in the Scaffold+ADMScs group, moderate in Scaffold group and weak in the Suture thread group (Fig. 4).
Figure 4. Photomicrographs of Control (a, b), Suture thread+ADMSCs (c), Scaffold (d) and Scaffold+ADMSCs (e, f) groups. (a), (b) Normal urothelium composed of 2–3 layers: a basal cell layer, an intermediate cell layer, and a superficial or apical layer composed of umbrella cells; lamina propria layer composed by collagen fibers and smooth muscle; detrusor layer (muscle layer). (c) Moderate angiogenesis, inflammatory infiltrate and giant cell reaction processes, and weak collagenization in the urinary bladder. (d) Moderate angiogenesis and collagenization processes, and weak inflammatory infiltrate and giant cell reaction in the urinary bladder. (e), (f) Normal urothelium; intense angiogenesis and collagenization processes, and weak inflammatory infiltrate and giant cell reaction in the urinary bladder. Hematoxylin-Eosin: a, c, f. Masson’s trichrome: b, d, e.

All of these data are summarized in Table 1. In in vivo trials the GO/6 ARM-PEG-amine/PCL and adipose mesenchymal stromal cells sewed on bladder showed complete healing after of surgical bladder injury treatment. The histopathological analyses showed the presence of healing cells, as
compared with the previous surgery injuries. After cell adhesion it was found a high bladder healing area.

Histological grades for angiogenesis, inflammatory infiltrate, giant cell reaction and collagenization processes; absent - 0; weak - 1; moderate - 2; intense - 3.

Table 1. Histopathological evaluation of the angiogenesis, presence of inflammatory infiltrate, giant cell reaction and collagenization in the urinary bladder of the rats from different experimental groups.

4. Conclusions

Total characterization of GO/6 ARM-PEG-amine film was carried out. This optimized platform exhibited growth and adhesion of adipose-derived stem cells (ADMSC) and excellent properties for the wound healing processes. The trials to made the adhesion of adipose mesenchymal stromal cells was confirmed by LIVE and DEAD®. In in vivo trials the GO/6 ARM-PEG-amine/PCL and adipose-derived stem cells sewed on bladder showed complete healing after of surgical bladder injury treatment. The histopathological analyses showed the presence of healing cells, as compared with the previous surgery injuries. After cell adhesion it was found a high bladder healing area. Data suggested a great potential of GO/6 ARM-PEG-amine/PCL films as platform to adipose-derived stem cells, as well as, new material for treatment several urological diseases. Improved organized muscle bundles and urothelial layer were also observed in animals treated with GO/6 ARM-PEG-amine/PCL scaffold with adipose-derived stem cells compared with those treated only wit Suture thread or Scaffold. Thus, our biomaterial could be suitable for tissue engineered urinary tract reconstruction.

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