In vivo nanotoxicological profile of graphene oxide

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Abstract. Graphene oxide, a widely studied nano materials, exhibits numerous beneficial effects in medical devices. The graphene sample Graphene oxide (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 this study. Characterization of GO and stability were previously studied. All the assays were carried out at a concentration of 1 mg/mL (stock suspension by ultrasonication)(stable 10 days). In this study the first parameter evaluated was in animal toxicity in acute and chronic responses. The second one was to observe the morphology and histological changes on major organs, like signs of inflammatory areas. A total of 25 rats (Fischer 344) were divide in 5 groups (n = 5 animals): negative control group (NCT); Positive cancer control group (PCCT); GO group-1 (GO 1); GO group-2 (GO 2) and GO group-3 (GO 3), with different concentrations of graphene oxide. All the groups were treated by via intra peritoneal (i.p) administration. Biochemical and histopathology results of acute toxicity showed no alterations. In the chronic toxicity was found deposit sections and morphology alterations in different tissues (inflammations). Genotoxicity was dose dependent of GO. This study gave us the limit concentrations of GO free for any biological study.

1. Introduction

Graphene oxide one of derivatives of graphite, in the last years has become a promising candidates for important biomedical applications. Due at its characteristics it can be used at base to material which will be used with biological functionality [1-3]. The comprehension of the toxicity of these materials has been highlighted in numerous studies [4,5].

One of the concerns in the use of these nanomaterials is how these structures could influence the cellular environment where they were inserted, There are studies that indicate a correlation between the methodology used in its synthesis and chemical composition with the cytotoxic effects [6,7]. Study by Paul and Sharma [8], assessed the platelet action or hemolysis of graphene during growth and differentiation in stem cells. The authors concluded that the graphene does not produce platelet aggregation and induction of hemolysis when did not exceed 0.1%, being permitted in the case of medical materials is at most 1%. In another study performed in 2011[7] using normal human lung cells (BEAS-2B) could be observed toxicological aspects cytotoxicity and apoptosis induced by

evaluating graphene by 3- (4,5- dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) after 24 and 48 h of exposure. There was a significant decrease in cell viability dependent on the concentration and exposure time and increase of early and late apoptotic cells compared to control cells. Direct responses in hemolytic action or platelet in the presence of aqueous graphene concentrations 1.0 mg/mL, 5.0 mg/mL and 7.0 mg/mL were observed [6], showing the importance of knowing the nanomaterials and their concentrations without toxic effect when they are being used in biological systems. Then, the aim of this work is to study the *in vivo* toxicity of graphene oxide.

2. Methods

2.1. Preparation of graphene oxide suspensions (GO)

Preliminary studies have shown that it is possible to obtain stable suspensions of GO in deionized water (1mg/mL) for up to 10 days. The suspensions of graphene oxide (1.0 mg/mL) was prepared in sterile deionized water and conditioned in 15 mL Falcon tubes (sterile) and placed in ultrasonic bath for 30 minutes.

2.2. Analysis of genotoxicity of GO sample: micronucleus test

The micronucleus test is a widely used method for monitoring of genotoxic damage in populations exposed to mutagenic and carcinogenic substances. The frequency of micronuclei observed at a given time can be considered a complex response between genotoxic activity and efficiency of the physiological defence mechanism of the test organism [9,10].

A total of 25 rats of the Fischer 344 strain were divided into 5 groups (5 animals per group): Group Negative Control (NC): received a dose of 0.3 mL of 0.9% saline intraperitoneally (i.p); Positive Control Group (PC): received a dose of 1.5 mg/mL of N-methyl-N-Nitrosourea (MNU - Sigma, St. Louis, MO, USA)(i.p.); Graphene Oxide Group 1 (GO 1): received a dose of 0.05 μg/g of GO (i.p.); 2 graphene oxide (GO 2): received a dose of 0.5 μg/g of GO (i.p.); 3 graphene oxide (GO 3): received a dose of 5.0 μg/g of GO (i.p.).

The animals were killed 24 h after the administration of MNU. Femurs were removed and cleaned; and epiphysis was sectioned to expose the spinal canal. The bone marrow was aspirated with a 1 ml of foetal bovine serum (FBS) and placed in Falcon tube containing 2 mL of FBS. The bone marrow was resuspended several times to form a suspension with FBS. Subsequently, the samples were centrifuged at 1000 rpm for 5 min. The supernatant was removed and the precipitate was carefully aspirated with a pipette. Then 100 μL of the precipitate of each sample were applied in histological slide and smear done with the aid coverslip. The slides were smears with drying for 24 h at room temperature. After this period, the samples were fixed in absolute methanol for 15 min and stained with Giemsa (1:10 dilution in pH 6.8 sodium phosphate buffer).

The samples were analysed in light microscope with 100X immersion objective and the number of micronuclei will be counted in 3.000 polychromatic erythrocytes (PCEs) per animal. The micronucleus identification followed the criteria described by Fenech [11]:

a) micronucleus should be one third smaller than the core;
b) a micronucleus should not touch the main core and;
c) a micronucleus should not refract, or should have the same colour and intensity of the main core.

2.3. Histopathological analysis

For the analysis of systemic toxicity after 30 days of treatment, all animals will be anesthetized with xylazine / ketamine (1: 1) and euthanized. The bodies will be collected (spleen, liver and peritoneum) and fixed with Bouin liquid for 12 hours. Tissues follow standard fixation procedures, dehydration, and inclusion diafanization using Paraplast Plus (ST. Louis, MO, USA). The materials will be 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) [12].

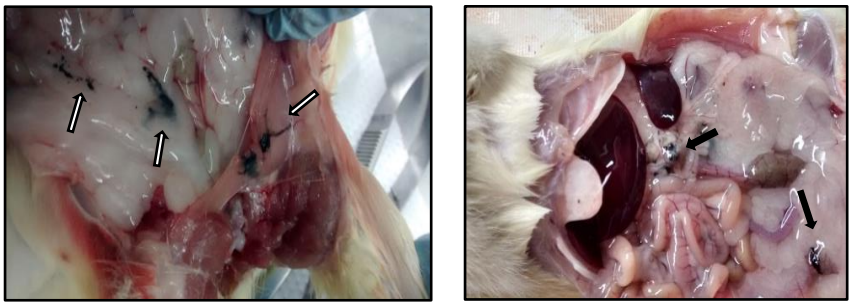
2.4. Biochemical analyses

The animals were anesthetized and before the euthanasia the blood samples were collected by cardiac puncture (left ventricle). Serum was separated by centrifugation at 6000 rpm, 5 min, and used to investigate the possible hepatotoxic effects, nephrotoxic, cardiotoxic of different GO concentrations through analysis: TGO (transaminase glutamic oxaloacetic) and TGP (transaminase glutamic pyruvic transaminase), a specific marker for injury hepatic parenchyma. Circulating levels of urea and creatinine were performed to check kidney function. The measurement were performed by the 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).

3. Results and Discussion

3.1. Deposition of GO in application sites

During the sacrifice were found sites of GO deposition (regions where the doses were applied) and GO concentration in the peritoneum, especially in the group received GO suspension at higher dose (5.0 µg /g) (Figures 1 A and B).



(A) (B)

Figure 1 AB. Exposure of abdominopelvic cavity in the animals from GO 5.0 µg/g group. Presence of dark agglomerates (arrows) in the peritoneum.

3.2. Systemic toxicity

The systemic toxicity was correlated with the degree of inflammation in organs: the peritoneum, liver and spleen. The degree of inflammation was assessed by a semi-quantitative scale. The inflammation was scored as: 0, absent inflammation; 1, weak inflammation (less than five lymphocytes within a grid 6200 to 10610, an area of 0.25 mm^2; 2, moderate inflammation (inflammatory mononuclear cells scattered throughout the tissue, but the stroma still clearly visible); 3, severe inflammation (mononuclear inflammatory cells infiltrating the stroma thick) (modified from [13] (Table 1).

3.2.1. Systemic Toxicity - Histophatologycal Results

Spleens, livers and peritoneum of the control groups showed no inflammation, hemosiderin deposits and GO deposits. In the group which received 0.05 µg/g GO can be seen that spleens inflammation was absent in 40% and 40% of the animals showed mild inflammation with hemosiderin deposits. At the peritoneum, although there specific areas of GO deposition, 80% of the animals showed mild inflammation, but there was no evidence of deposition in this group. Similar to the control group, the

liver of did not show inflammation and deposition of hemosiderin, but 20% of the animals showed GO deposition in the liver parenchyma. In the group receiving 0.5 µg/g GO can be observed intense inflammation hemosiderin deposits in the spleen and GO 40% of the animals. At the peritoneum, 80% of the animals showed mild inflammation, 60% of the animals showed deposition of GO. In the liver of the animals 20% of this group exhibited mild inflammation, and a deposition GO occurred in 60% of animals. The worst result occurred in the group receiving 5.0 µg/g of GO, as expected. The spleen was exhibited intense inflammation in 100% of animals, and 60% hemosiderin deposits and deposition of GO in 80% of animals. They presented peritoneum intense inflammation and deposition of GO in 100% of animals. In the liver, 10% of the animals showed mild inflammation and in 80% of animals showing deposition of GO. Some pictures of these results can be seen in figure 3.

Table 1: Degree of inflammation in the spleen, liver and peritoneum of the animals from different experimental groups (n=5).

Tissue	Groups																			
	Control					GO 0.05 µg/g					GO 0.5 µg/g					GO 5.0 µg/g				
Number of animals	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Spleen	0	0	0	0	0	1	0	2^{a}	2^{a}	0	3^{a}	$3^{a,b}$	2	2^{b}	1	3^{b}	3^{b}	3^{a}	$3^{a,b}$	$3^{a,b}$
Liver	0	0	0	0	0	1	1	1^{b}	1	1	1^{b}	1^{b}	1	1^{b}	1	1	1^{b}	1^{b}	1^{b}	1^{b}
Peritoneum	0	0	0	0	0	1	1	1	1	0	2^{b}	2^{b}	2	2^{b}	1	3^{b}	3^{b}	3^{b}	3^{b}	3^{b}

Note: 0, absent inflammation; 1, weak inflammation; 2, moderate inflammation; 3, intense inflammation. a hemosiderin agglomerate. b GO agglomerate.

3.3. Genotoxicity

The number of micronucleus was significantly higher in the group of animals Positive Control (induced using a single dose of 1.5 mg/mL/rat body (7.5 µg/g) of N-methyl-N-nitrosourea (MNU)) compared to other experimental groups. The micronucleus number of animals in the GO 5.0 µg/g was significantly higher compared to GO groups 0.5 µg/g, GO 0.05 µg/g and Negative Control (without any treatment), which were not statistically different (Table 2). Still, they were observed in all concentrations, GO agglomerates from polychromatic erythrocytes and micronucleated polychromatic erythrocytes, indicating the migration of the peritoneum compounds (application site) to the bloodstream (Figure 4). The results indicate that the higher the concentration of GO, the greater the genotoxicity.

3.4. Biochemical analysis

The results showed that serum levels of TGO, TGP, urea and creatinine in treated animals 24 h before with GO 0.05µg/g, GO 0.5 µg/g and GO 5.0 µg/g did not differ significantly between itself, as well as the control group (Table 3). These results demonstrated that the GO solutions in concentrations showed no acute toxicity in the liver and kidneys.

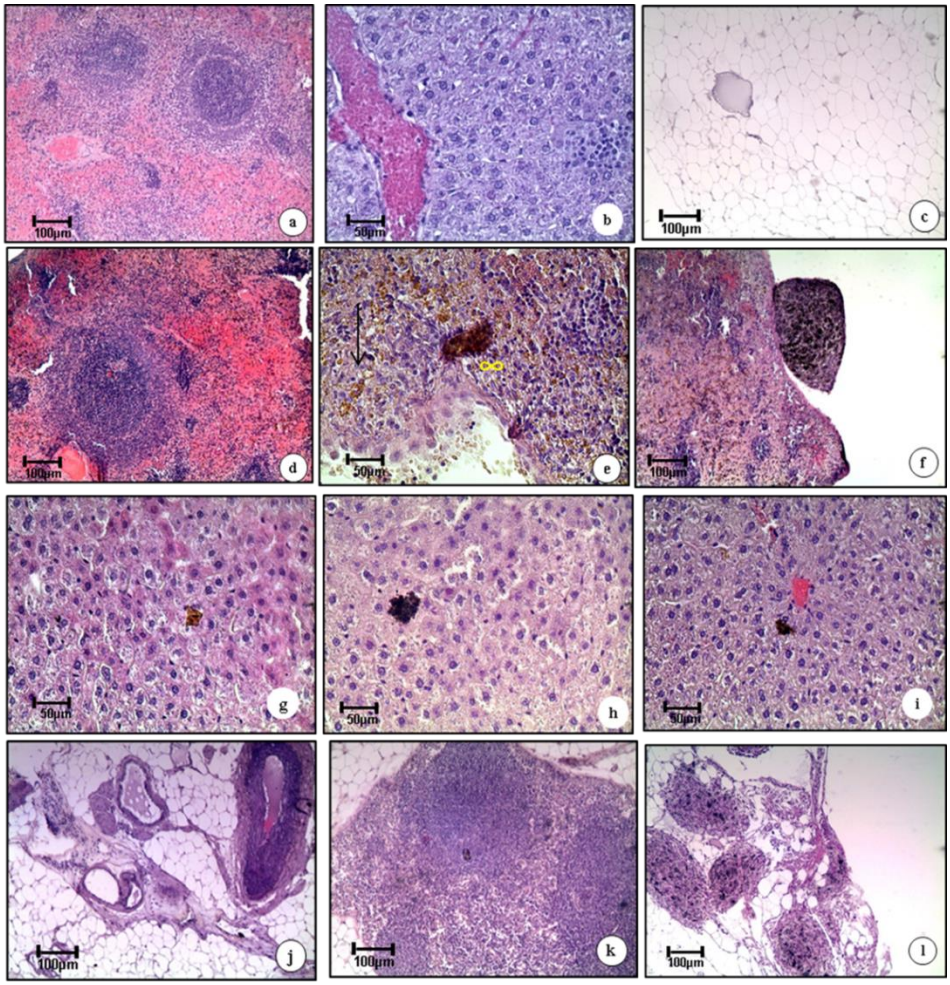


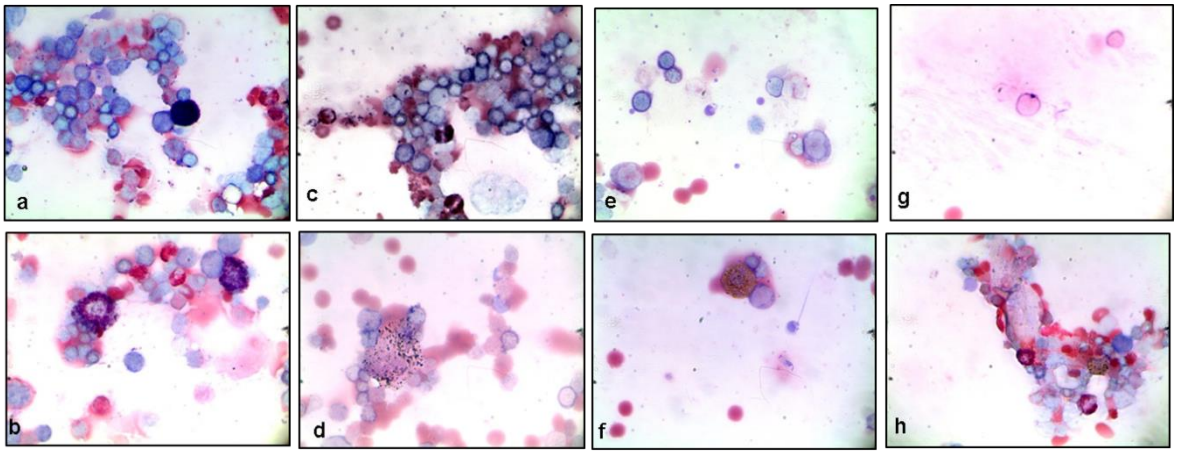
Figure 3. Photomicrographs of the spleen, liver and peritoneum of the animals from Control (a, b, c), GO 0.05 µg/g (d, g, j), GO 0.1 µg/µL (e, h, k) and GO 5.0 µg/g (f, i, l) groups. (a), (b), (c) Spleen (a), liver (b) and peritoneum (c) showed no signs of inflammation, as well as hemosiderin and GO agglomerates. (d) Spleen showed moderate inflammation. (e), (f) Intense inflammation in the spleen with GO and and hemosiderin agglomerates. (g), (h), (i) Weak inflammation was observed in the liver. (j) Peritoneum showed moderate inflammation. (k), (l) Intense inflammation in the peritoneum with GO agglomerates.

Table 2: Average of micronuclei number in relation to 3.000 polychromatic erythrocytes in the different experimental groups.

Groups	Number of Polychromatic erythrocytes	Average Micronucleus
Negative Control (n=5)	3,000	10 a
Positive Control (n=5)	3,000	283 b
GO 0.05 µg/g (n=5)	3,000	10 a
GO 0.5 µg/g (n=5)	3,000	26 a
GO 5.0 µg/g (n=5)	3,000	34 c

Different lowercase letters indicate statistically significant difference ($P < 0.01$) between groups after the Tukey test.

Figure 4. Photomicrographs of polychromatic erythrocytes and micronucleated polychromatic erythrocytes of the animals from Negative Control (a), Positive Control (b, c), GO 0.05 µg/g (d, e), GO 0.5 µg/g (f, g) and GO 5.0 µg/g (h, i) groups.



3.5. Biochemical analysis

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Table 3. Biochemical parameters of liver and renal functions 24 hours after intraperitoneal administration of GO 0.05 µg/g, GO 0.5 µg/g and GO 5.0 µg/g.

Samples	TGP ($U\ L^{-1}$)	TGO ($U\ L^{-1}$)	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 (0.05 µg/g)	31.0 ± 2.8	53.5 ± 2.1	31.5 ± 0.7	0.31 ± 0.06	$P < 0.01$
GO (0.5 µg/g)	32.5 ±3.5	53.0 ± 2.8	27.0 ± 2.8	0.26 ± 0.03	$P < 0.01$
GO (5.0 µg/g)	27.7 ± 2.0	55.2 ± 1.6	32.06 ± 0.6	0.28 ± 0.04	$P < 0.01$

TGP (transaminase glutamic pyruvic transaminase) and TGO (transaminase glutamic oxaloacetic).
Data were expressed as mean ± standard deviation (n = 05)
None of the average values differ by Tukey test.

4. Conclusions

Evaluation of the systemic toxicity of GO solutions showed signs of inflammation in the spleen at the lowest concentration of GO (0.05 µg/g); intense inflammation, concomitant with hemosiderin regions were detected in the spleens of animals receiving doses from 0.5 µg/g and 100% of the animals was detected intense inflammation and 60% hemosiderin group recioving 5.0 µg/g. Also in 80% of the animals of this group deposition of GO was observed.

In relation to the liver, there were no signs of inflammation or deposition GO in the control group and GO of 0.05 μg/g; although, in GO groups 0.5 μg/g and 5.0 μg/g mild inflammation was observed. Deposition of GO was observed from the GO 0.5 μg/g in a considerable number of animals (60%) and even more significant in the GO group of 5.0 μg/g (80%). When in the peritoneum higher concentration of GO was applied, greater inflammation was observed. 80% of animals which received 0.05 μg/g showed mild inflammation, and 60% GO deposition in animals When they received GO 0.5 μg/g a 100% of animals exhibeted inflammation and GIO deposition. At GO 5.0 μg/g a 100% exibited high inflammation and large GO deposition.
Regarding the genotoxicity of GO solutions it was verified that the counting of micronucleus of the positive control group (MNU) was significantly higher than the other experimental groups, and the group received GO 5.0 μg/g, compared to other groups (GO 0.5 μg/g, and negative control) also showed a greater number of micronucleus. It was also possible to notice the presence of GO between erythrocytes indicating migration from GO peritoneum (application site) to the bloodstream.
Through biochemical tests can be verified that there was no change in hepatic or renal functionality of animals that received different doses of GO, 24 h after this administration. These data demonstrated that no impairment in this study was observed. Then, it could be concluded that GO of dose less than 0.5 μg/g failed to exhibit systemic toxicity and mutagenesis, while the dose of more than 5.0 μg/g exhibited obvious toxicity and showed potential to cause mutagenesis.

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