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ORIGINAL ARTICLE

Toxicity study of graphene-coated Poly(methyl methacrylate) membranes on the brain cortex of rats

Eleni TSIANAKA [1], Evangelia SERETI [2], Constantina PAPACHARALAMBOUS [3], Maria IOANNOU [3], Nikolaos PITSIKAS [2], Athanasios DIMOULAS [4], Nikos SAKELLARIDIS [2,5], Kostas FOUNTAS [1], Konstantinos DIMAS [2,5]

[1] Department of Neurosurgery, University Hospital of Larissa, University of Thessaly, Larissa, Greece
[2] Department of Pharmacology, Faculty of Medicine, University of Thessaly, Larissa, Greece
[3] Department of Pathology, Faculty of Medicine, University of Thessaly, Larissa, Greece
[4] National Center for Scientific Research "Demokritos", Athens, Greece
[5] Clinical Pharmacology, University Hospital of Larissa, University of Thessaly, Larissa, Greece

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ABSTRACT
Graphene is a material, which has attracted great attention of the scientific community in several fields of biomedicine, neurosciences being one of the fields holding great interest for the application of graphene-based materials and devices. Our study aimed to determine the *in vivo* brain tissue reaction and to study the possible impairment of memory in a long-term Graphene exposure. Towards this aim we tested the toxicity of graphene membrane in the form of few layers (few layers graphene, FLG) implanted on the frontal brain cortex of adult Wistar rats after careful durotomy. The results from this study advance our knowledge on graphene *in vivo* toxicity in CNS and suggest that the application of FLG as a patch on the brain cortex seems to be quite safe under the experimental conditions tested, herein as no change in locomotor activity of the rats or major histopathological reactions of the brain to the material were observed.

KEY WORDS: few layer graphene; toxicity; brain; rats

Introduction

Graphene, a nanosized material of carbon allotrope has attracted much attention in the fields of physics, chemistry, biology and medicine, and their related interdisciplinarities with applications ranging from biosensors to diagnosis and from tissue engineering to drug delivery and cancer therapy. Transparency, durability, elasticity and high conductivity make graphene an attractive material for applications also in the field of neurosciences (Bitounis *et al.*, 2013; Kuzum *et al.,* 2014). Generally, graphene-based nanomaterials are classified into several types such as graphene oxide, reduced graphene oxide and graphene with varied layers (Yao *et al.,* 2012), which types also show variability in their physicochemical and biological properties. The majority of the biomedical studies using graphene-based nanomaterials have focused on graphene oxide, while there are only a limited number of biological studies on graphene itself (Orecchioni *et al.,* 2016). The number of layers of graphene is important because it determines the characteristics of the bioactive surface. It is expected that the adsorption capacity of biological molecules decreases significantly as the number of layers increases. For Few Layers Graphene (FLG), the thickness of every single layer is 0.34 nm. FLG consists of 2 to 10 graphene layers. Characteristics determining its biological activity are chemical purity, the bio-exposed surface, the number of layers, the cross-section and the chemical characteristics of the surface. Generally, nanoparticles sized less than 35 nm cross the blood-brain barrier (BBB), less than 40 nm can enter the nucleus and less than 100 nm can enter the cell (Mytych & Wnuk, 2013).

A crucial concern for biomedical applications is the assessment of the toxicity of the materials to be used. Despite their tremendous applications and potential in biomedicine, these materials are not devoid of toxicity.

Correspondence address:
Assoc. Prof. Konstantinos Dimas, PhD.
Department of Pharmacology,
Faculty of Medicine, School of Health Sciences
University of Thessaly
Panepistimiou 3 (Biopolis), 41500 LARISSA, Greece
E-MAIL: kdimas@med.uth.gr

Pristine graphene (pG) for example has been shown to exhibit toxic effects in chicken embryos and impair DNA synthesis especially in the brain of the embryos (Sawosz *et al.,* 2014). The toxicity of graphene has been extensively studied and debated in the literature. Results show that the toxicity of graphene is dependent on several factors such as shape, size, purity, post-production processing steps, oxidative state, functional groups, dispersion state, synthesis methods, route and dose of administration, and exposure times (Lalwani *et al.,* 2016).

Regarding FLG *in vitro* exposure of nerve cells to 10 μg/ml FLG, has been shown to increase the levels of reactive oxygen species (ROS) and mitochondrial lesions after 4 and 24 hours (Zhang *et al.,* 2010). Creation of intracellular ROS is the predominant mechanism of Graphene materials cytotoxicity. Other *in vitro* studies saw unaffected electrical activity and neuronal viability after exposure to Graphene (Li *et al.,* 2011, Fabbro *et al.* 2016, Bramini *et al.* 2016). Furthermore, the exposure of healthy neurons to a Graphene scaffold resulted in their development and the creation of synapses (Fabbro *et al.,* 2016). A short term (7 weeks) *in vivo* study, using colloidal Graphene coated on electrospun microfiber scaffolds, saw suppression of glial process in rat brain, which is very promising in brain injury cases (Zhou *et al.,* 2016). There are several studies focusing on *in vitro* study of Graphene but *in vivo* study is still deficient.

However, as it is clear from discussed above, very few studies are known for the toxicity of graphene placed directly in the brain of animal models for use *e.g.* in tissue engineering for brain injuries or depot formulations for the local administration and release of drugs. In this context we herein aimed to study the *in vivo* brain tissue reaction and the possible impairment of memory in long-term exposure to graphene in the form of FLG. Towards this purpose we performed durotomy and directly placed an FLG membrane on PMMA in the brains of adult Wistar rats.

Materials and methods

Materials

The chemical vapor deposition method was chosen to produce Graphene because it allows the easy transfer of material to different substrates, while produced FLG has not many defects (Miao *et al.,* 2011; Yi *et al.,* 2013). In our study, FLG was transferred on Poly (methyl methacrylate) (PMMA 495K dissolved in anisole at a thickness of 170 nm, Microchem, Newton, USA)), which is already used in different neurosurgical procedures (*e.g.* cranioplasty). FLG was produced by the Epitaxy and Surface Science (ESSL) laboratory, National Center for Scientific Research "Demokritos" (Athens, Greece). FLG was sterilized by putting the material into Phosphate buffer saline (PBS, Thermo Fisher Scientific, Waltham, USA), which was further heated at 120 °C under 1 bar pressure for 30 minutes, conditions that did not result in any changes of the FLG properties.

Durotomy and FLG placement on the brain of Wistat rats

Male 3-month-old albino Wistar rats (Hellenic Pasteur Institute, Athens, Greece) that weighted 250–300 g were used in this study. The animals were housed in Makrolon cages (47.5 cm length × 20.5 cm height × 27 cm width) three per cage, in a climate-regulated environment (21±1 °C; 50–55% relative humidity) under a 12 h/12 h (lights on at 7:00 AM) light/dark cycle with free access to food and water. All experiments were approved by the IACUC of the Faculty of Medicine, University of Thessaly and experimental procedures and handling of animals were conducted in accordance with the Greek laws (PD 56/2013 and Circular 2215/117550/2013) and to the guidelines of the European Union (2013/63/EU).

Two groups of rats were formed. In Group A we made all the procedures without using Graphene (control/sham group). In Group B we used the Graphene. Every group included 3 rats. All rats went under pre-surgical medical control and measurement of different parameters (age and weight). Only healthy animals were included in our study. One hour before surgery, 0.05% buprenorphine as an analgesic was administered.

Anesthesia was achieved by administration of mixture Ketamine (100 mg/ml)/Xylazine (20 mg/ml) at a dose of 1500 μl/200 gr of animal's weight. Animals were in complete anesthesia after about 3 minutes and anesthesia had a duration of 1–2 hours. Craniotomy took place according to the guidelines of Comparative Medicine and Animal Resources Center – SOP 202.01. (steps 4.2.2, 4.2.5–4.2.6, 4.2.8–4.3.9, 4.3.11 and 4.3.17) (Gourdon & Jimenez, 2016). The material was placed on the frontal brain cortex, after careful durotomy. Post-operative management was indicated, including subcutaneous Buprenorphine 0.05 mg/kg 2 times daily. Animals were observed for signs of pain or anxiety until they returned to their normal preoperative routine and behavior.

Locomotor activity

Spontaneous locomotor activity took place three months after the placement and was assessed in an activity cage (Ugo Basile, Varese, Italy). The apparatus consisted of a box made of Plexiglass (41 cm length × 33 cm height × 41 cm width). Both horizontal and vertical activities were recorded. The test was performed as described by Grivas *et al.* (Grivas *et al.,* 2016). On the test day, rats were transported to the test room and left in their home cages for 2 h. Thereafter, each animal was placed into the locomotor activity arena and spontaneous locomotion was recorded for 5 min. Experiments were conducted between 9:00 AM and 3:30 PM during the light phase of the light/dark cycle. To avoid the presence of olfactory cues, the apparatus was thoroughly cleaned with 20% ethanol and then after each trial wiped with dry paper.

Histopathological analysis

Three and a half months after the procedure, animals were euthanized using CO_2 and their brains were removed, [following a standard protocol (Paul *et al.,* 2008)] for further study. Tissue sections from sampled brains were fixed in

10% buffered formalin for 24 hours and were processed to paraffin blocks in an automate tissue processor (Shandon Citadel 2000, THERMOELECTRON corporation, UK). Three μm thick sections were obtained using a Leica TP1020 microtome and they were routinely stained with Hematoxylin and Eosin stain. Stained slides were subsequently examined under a light microscope.

Statistics

Data are expressed as mean ± S.E.M. and were evaluated by the Student's t-test. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

We studied FLG on PMMA substrate delivered directly on the frontal lobe cortex of the rats' brain. Post-surgical period was normal for all studied animals with no indications of toxicity or other pathological signs.

Spontaneous locomotor activity test results indicate that graphene did not induce any change in horizontal or vertical motor activity of rats (Table 1).

Total histological findings are summarized in Table 2. Histologically, the examined tissue specimens regarding Group A showed no particular changes. Among specimens from the graphene group (Group B) only one sample (rat 3) showed slight mononuclear infiltration in the subarachnoid region (Figure 1a). This sample also showed marked inflammatory infiltration with foreign-body tissue reaction most probably to suture (Figure 1b). There have been detailed descriptions of suture reactions in different studies related with the biocompatibility of various sutures materials (Anderson *et al.,* 2008; Selvi *et al.,* 2016). In our study, we used polypropylene 3-0 suture for monolayer skin closure. For that kind of material, foreign body reactions have been described previously (Anderson *et al.*, 2008) and the secondary histological findings, such as subarachnoid oedema and fibrin deposition observed in this animal, might be explained in the context of this particular tissue reaction. There was an amorphous substance remaining focally in the area where the Graphene was administered in rat 1. This specimen showed an oedematous subarachnoid region with fibrin deposition without inflammatory infiltration (Figure 2). These are typical findings for traumatic subarachnoid hemorrhage caused during placement procedure (Suzuki *et al.,* 1977; Gaberel *et al.,* 2014). The cerebrum of the third rat of group B (rat 6) showed no remarkable changes (Figure 3).

Graphene [single graphite layer, consisting of a hexagonally arranged, sp2 bonded, stable two-dimensional allotrope of carbon with a plethora of unique properties (Novoselov *et al.,* 2004)] and graphene oxide have been extensively studied as some of the most promising biomaterials for biomedical applications due to their unique properties: two-dimensional planar structure, large surface area, chemical and mechanical stability, higher conductivity and good biocompatibility. Due to their advantageous properties, graphene and GO have recently emerged as new and competitive drug delivery systems with the potential to be applied for systemic targeting and local drug delivery systems. Before any clinical studies can be designed and begin, the successful design of graphene and graphene oxide-based systems requires to address some important issues for a biomedical application such as the modifications required to build an efficient carrier able to release drugs in controlable manner, but at the same time, it is required to address their biocompatibility and toxicity**.**

Graphene nanosheets' biocompatibility is still controversial. This is due to the high heterogeneity of materials present on the market and the large variety of synthesis methods that affect, to various extents, its interaction with the biological systems. Two studies have already pointed out that graphene nanosheets may be harmful for the environment and human health, resulting thus to a debate about their use in biomedical applications (Bramini *et al.,* 2016; Reina *et al.,* 2017). According to Amrollahi-Sharifabadi *et al.* (Amrollahi-Sharifabadi *et al.* 2018) GO nanoplatelets (GONs) in particular, induce inflammation and granulomatous reaction in various

Table 1. Effects of Graphene on rats' performance in a locomotor activity test. Group A, sham rats; Group B, rats that received graphene membrane.

Group	Horizontal activity (Counts/5 min)	Vertical activity (episodes/5 min)
A	1270.7±158.4	30.7±1.3
B	1359±106.3	32±2.1

Table 2. Detailed histopathological findings for every rat of the two groups A and B. Group A, sham rats; Group B, rats that received graphene membrane.

Group	Rats	Post-surgical observation	Histopathological findings	Conclusion
A	2	Normal	No remarkable changes.	No pathological findings
A	4	Normal	No remarkable changes.	No pathological findings
A	5	Normal	No remarkable changes.	No pathological findings
B	1	Normal	Amorphous substance remaining focally where FLG was administered. Oedema and fibrin deposition in the suburachnoid region	Chronic traumatic subarachnoid hemorrhage
B	3	Normal	Mononuclear infiltration in the subarachnoid region	Suture reaction
B	6	Normal	No remarkable changes.	No pathological findings

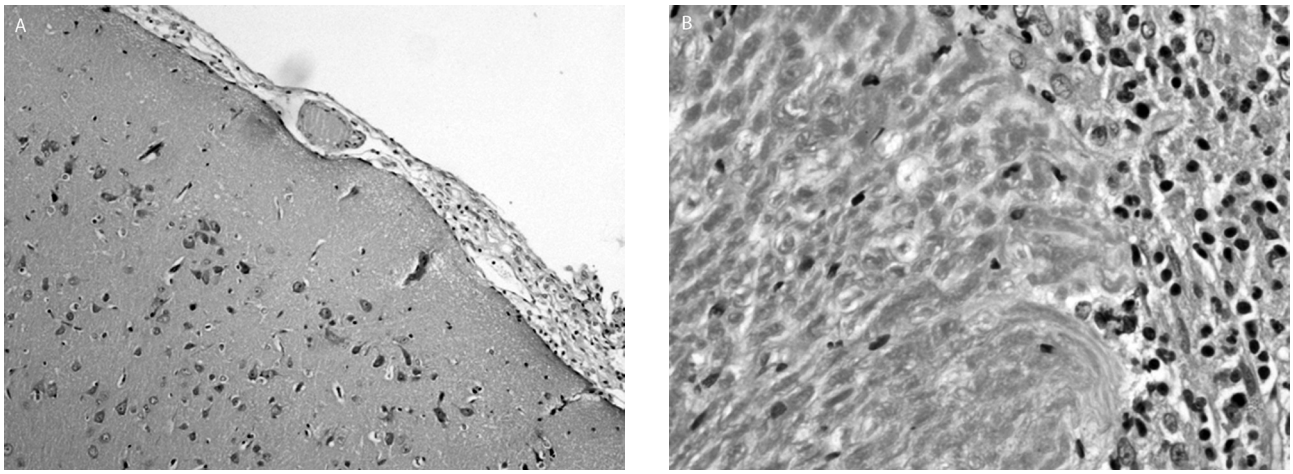

Figure 1 (a). Histology shows mononuclear inflammatory infiltration in subarachnoid region. Hematoxylin and Eosin stain, original magnification ×10. **(b).** Histology shows foreign body reaction to suture. Hematoxylin and Eosin stain, original magnification ×40. Images are representative of rat no 3, group B.

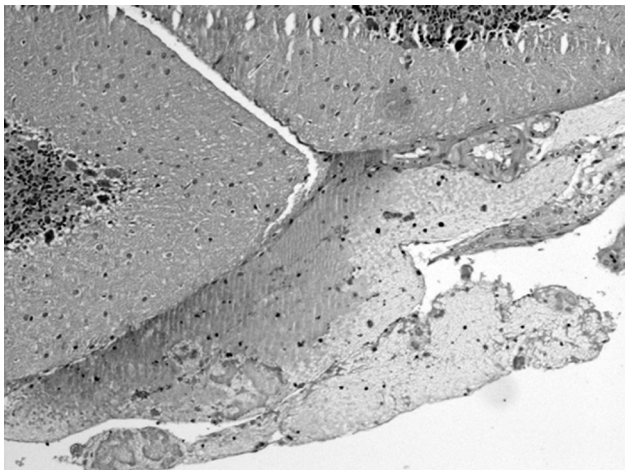

Figure 2. Histology shows oedema and fibrin deposition in an area with amorphous substance in the surface of brain. Hematoxylin and Eosin stain, original magnification ×10. Images are representative of rat no 1, group B.

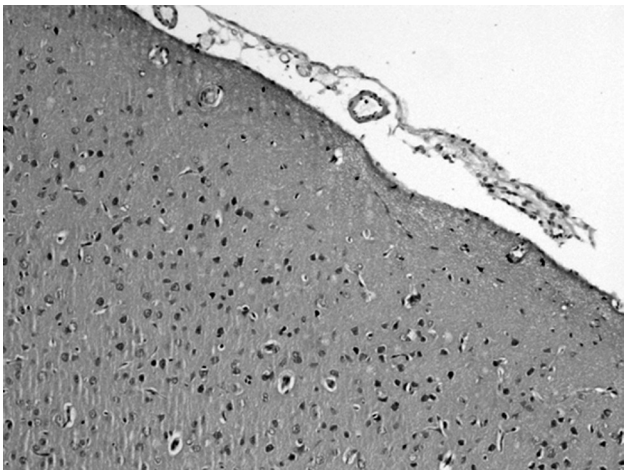

Figure 3. Histology shows normal brain tissue without inflammation. Hematoxylin and Eosin stain, original magnification ×10. Images are representative of rat no 6, group B.

organs, depending on the given dose, because of accumulation in the organism after intraperitoneal injections of the material. Based on various studies, GO appears to have the most severe inflammatory effect for the lungs among different graphene-based materials in respiratory exposure (*in vivo*) studies, from which FLG appears to have the safest profile (Duch *et al*. 2011, Schinwald *et al*. 2014, Park *et al*. 2015, Roberts *et al*. 2016, Mao *et al*. 2016, Ema *et al*. 2017, Lee *et al*. 2017, Bengtson *et al*. 2017).

In this study using the chemical vapor deposition we have produced FLG on a PMMA substrate. This procedure is considered inexpensive and produces high-quality monolayer and few-layer graphene with a low number of defects (Miao *et al.,* 2011; Yi *et al.,* 2013).

FLG is considered a very promising material in the field of biomedicine. Recently in a nice study Russier *et al.* (Russier *et al.* 2017) showed that FLG dispersions could kill specifically the monocytes in a myelomonocytic leukemia model, displaying neither toxic nor activation effects on the other cells of the immune system. Importantly the authors tested the toxicity of this FLG dispersion in C57BL/6 mice after intraocular delivery and found it safe.

Obviously, any further exploitation in the central nervous system of biomedical devices requires deep knowledge of their interactions with the neuronal tissue, toxicity being among the most important. I*n vitro* and *in vivo* toxicological studies have evaluated the interactions of graphene-based nanomaterials with various living systems and have examined the short- and long-term *in vivo* toxicity and biodistribution of various types of graphene (for a detailed review on the toxicology of graphene see Lalwani *et al.,* 2016). Toxicity of graphene derivatives has been tested in small and large animal models (mice and rats) under various modes of administration: intravenous, oral, pulmonary, intraperitoneal and even pharyngeal and intravitreal (Lalwani *et al.* 2016), though, with controversial results. Another form of the graphene materials (high biocompatible graphene quantum dots, HGQDs) has been administered *in vivo* showing a safe profile, even though it can cross the blood-brain barrier, promising

various interesting biomedical applications (Yan *et al*. 2020). However, the toxicity of graphene on the brain and especially in the form of FLG has not been reported so far. To our knowledge, only two studies have so far addressed the issue of graphene toxicity in the neural system but again not directly in the brain. One study tested reduced graphene oxide (rGO), seeing the safety of the material using it under the form of injectable dispersion in phosphate buffer saline, in a period of 30 days. The target for the injections was the adult mouse olfactory bulb (Deferal *et al.,* 2016). Other one studied graphene-polyelectrolyte multilayer onto electrospun poly-ε-caprolactone and the target was striatum of adult rats (Zhou *et al.,* 2016). Both studies showed no potential tissue toxicity. In this context, we next sought to study the toxicity of this device on the brain of living animals, in this case adult Wistar rats. The placement of FLG/PMMA membrane, after careful durotomy, was not found to result in any toxicity after more than 100 days showed by spontaneous locomotor activity tests and post mortem histological examination. A relevant study using ultra-trace concentrations of GO in the environment of zebrafish, shown significantly disturbed locomotor activity inducing Parkinson's-like symptoms (Ren *et al.* 2016). That study supports the idea of possible dose-related toxicity of graphene materials, as potential biohazards are based on the accumulation in the organism. At this point, we must underline the huge differences in the biological activity between the different types of graphene-based materials (Mytych & Wnuk, 2013) and that the toxicity of graphene is dependent on several factors such as shape, size, purity, post-production processing steps, oxidative state, functional groups, dispersion state, synthesis methods, route and dose of administration, and exposure times (Lalwani *et al*. 2016).

Concluding, our *in vivo* study is the first where the application of FLG as a patch on the brain cortex is studied regarding its safety. The results were indeed encouraging as the material was very well tolerated by the animals for more than 3 months. Extending studies, using larger cohorts and more specific applications such as the use for local drug release, would be of great interest in order of safety of FLG for use in CNS to be further certified, but its functionality as well to be tested.

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