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# Graphene oxide affects *in vitro* fertilization outcome by interacting with sperm membrane in an animal model

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ABSTRACT

We realized the exposure of boar spermatozoa to graphene oxide (GO) at concentration of 0.5, 1, 5, 10 and 50 μg/mL in an *in vitro* system able to promote the capacitation, i.e. the process that allows sperm cells to became fertile. Interestingly, we found that the highest GO concentration (5, 10 and 50 μg/mL) are toxic for spermatozoa, while the lowest ones (0.5 and 1 μg/mL) seem to significantly increase the sperm cells fertilizing ability ($p > .05$) in an *in vitro* fertilization experiment. To explain this finding, we investigated the effect of GO on sperm membrane structure (atomic force microscopy) and function (confocal microscopy and flow cytometry, substrate adhesion). As a result, we found that GO is able to interact with spermatozoa membranes and, in particular, it seems to be able to extract the cholesterol, which is a key player in spermatozoa physiology, from plasma membrane of boar spermatozoa incubated under capacitation conditions. In our opinion, these results are very important because they allow identifying either a plausible mechanism of GO toxicity on spermatozoa and new strategies to manage sperm capacitation.



## 1. Introduction

Graphene has recently received special attention in the research world due to its exceptional properties ranging from materials science to physics, electronics, optics and mechanics [1–4]. Graphene is a one atom thick molecule formed mainly of $sp^2$ hybridized carbon atoms. For this reason it is considered a two-dimensional material characterized by an exceptional strength, a high aspect ratio, an extraordinary thermal and electrical conductivity and a substantial transparency [2]. In the last five years a lot of studies have been performed on the capacity of some hydrophilic derivatives such as graphene oxide or reduced graphene oxide to favour stem cells differentiation [5–7], to induce synaptic connections in healthy primary neurons [8] as well as to strengthen or confer elasticity to biomaterials [9,10]. Preferential use of hydrophilic derivatives rather than pristine graphene in these studies is due to the fact that pristine graphene, despite its exceptional features, is difficult to disperse and process. Graphene oxide (GO) is instead promptly dispersible in water and, although it presents oxygen functionalities that affect the integrity of the honeycomb lattice of pristine graphene, keeps the majority of its properties.

The growing use of GO as biomaterial in recent years bring to light many important questions regarding its possible toxicity to organs, tissues and cells. In literature there are several papers addressing this issue, with specific regards to erythrocytes and fibroblasts [11], pheochromocytoma-derived PC12 [12], A549 [13], and human mesenchymal stem cells (hMSCs) [13,14]. In this context, one of the most interesting field of study would be the characterization of the GO exposure effect on reproductive function. This could have very important implications because of the key function of reproduction for all the organisms and because alteration on gametes could affect successive generations. Ultimately, the reproduction is the result of the interaction of gametes

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(the oocyte and the spermatozoa) within the female genital tract, where the spermatozoa complete the process that allows them to reach the ability to fertilize (the capacitation). Capacitation process takes from several hours to days, in which spermatozoa are exposed to a myriad of environmental factors and, eventually, exogeneous molecules able to modulate their physiological and biochemical machinery.

Indeed, a key point for biomedical applications of graphene derivatives and GO is their capacity to interact with biomolecules either via covalent and non-covalent interactions. In the former case covalent functionalization is mainly obtained by exploiting the ability of the enriched oxygen functional groups of GO to immobilize the biomolecules through nucleophilic substitutions or other similar reactions [15]. On the other hand, non covalent interactions such as $\pi-\pi$, van der Waals or electrostatic interactions may significantly affect the fate of these materials in biological systems including their cellular uptake and consequent biocompatibility [5]. Data are rather conflicting essentially due to the fact that all biological responses of GO vary depending on the number of layers of the investigated graphene, lateral size, hydrophobicity, surface functionalization, colloidal stability and concentration [16]. In particular, Yue et al. have investigated [17] the viability and cell responses of six different cell lines upon addition of GO of different dimensions. They discovered that only two phagocitic cell lines were able to internalize both nano- and micro-sized GO sheets and that almost no difference in terms of viability was detected in the six cells lines for concentration of GO lower than 20 μg/mL. In another study, the investigation of the interactions between GO and plasma membrane and the subsequent uptake of GO by macrophages, allowed to evidence [18] that large microsized GO sheets were prevailingly detected parallel and adsorbed to plasma membranes, while a high amount of smaller GO sheets were taken up by the cells after 6 h of incubation. From these examples, the interactions of GO with cell membrane appear to be crucial for investigating the toxicity and the effects of GO on cells. For this reason, and aware that virtually all the signalling systems involved in driving the communication of spermatozoa with female environment are located at membrane level, because male gametes are transcriptionally silent and their lipid metabolism is very limited [19,20], we thought it is noteworthy to investigate GO-spermatozoa interactions.

In this context the presence of GO could promote biologically relevant effects on male gametes, thus affecting the fertilization process. For this reason we studied the effect of GO on spermatozoa functional parameters during capacitation. In our work, we used an *in vitro* animal model because of the possibility to control all the environmental parameters and to work in the absence of ethical issues concerning manipulating animal gametes and embryos.

## 2. Results

### 2.1. GO characterization

In order to ascertain that the investigated GO could not be internalized in the cells we used micrometer sized GO, that demonstrated to interact prevailingly with the plasma cells without entering them [18]. In particular, intensity DLS data (Table 1) indicate a mean diameter of $800 \pm 30$ nm for the 0.5 μg/mL GO dispersions at 38.5 °C and the size do not increase on increasing the GO concentration, thus confirming the producer micrometer sized GO and its scarce tendency to aggregate at these concentrations. Polydispersity data evidence, as expected, the presence of a moderately polydispersed sample, ranging from 0.202 to 0.345. The irregular shape of GO flakes (see Fig. 1) contributes to the polydispersity of the sample. DLS measurements could not be performed in TMC199 due to the presence in the pure TMC199 of aggregates of micrometric dimensions (>5000 nm, data not shown). The obtained zeta potential changes between $-26 \pm 1$ at 0.5 μg/mL GO and $-54 \pm 1$ mV at 50 μg/mL in distilled water thus indicating a stable dispersion in which electrostatic repulsions keep GO flakes well dispersed. The presence of prevailingly single-layered or double-layered sheets of GO was verified by AFM measurements as evidenced by the high profile of Fig. 1. AFM images confirm the absence of obvious agglomeration in the sample.

### 2.2. GO cytotoxicity and acrosome damage

Since we used for the first time GO in a protein free system, firstly we assessed the concentration of GO able to promote a toxic effect on cell viability and on acrosome integrity. As it is shown in Fig. 2, the GO concentrations exceeding 5 μg/mL are directly toxic for spermatozoa, reducing their viability starting from the first hour of incubation. The effect on acrosome integrity starts to become statistically relevant ($p < .05$ vs. CTRL) at 5 μg/mL, while at lower concentration it appears to be negligible ($p > .05$ vs. CTRL).

### 2.3. Effect of GO on in vitro fertilizing ability of spermatozoa

For the first time, here, we assessed the effect of GO on fertilizing ability of spermatozoa on an animal model. As depicted in Table 2 it is evident a dose dependent-effect of GO.

In particular we have found the percentage of fertilized oocytes changed significantly ($p < .05$ vs. CTRL) at the different GO concentrations.

### 2.4. Functional characterization of GO interaction with sperm membranes

Under control conditions, sperm membranes become progressively more fluid (fusogenic) and as assessed by FRAP (see Fig. 3), the diffusion coefficient of DilC12 significantly increases (2.641 [1.330–5.260] vs. 6.505 [2.171–11.185] calculated diffusion coefficient (CDC) $cm^2/sec \times 10^9$, $p < .001$) (see Fig. 4 panel 1).

The presence of GO, at different concentrations, affected as well fluidity with values of 1.519 [1.023–3.444] at 0.5 μg/mL; 0.993 [0.015–4.5978] at 1 μg/mL and 4.556 [1.513–8.510] at 5 μg/mL $p < .001$) (see Fig. 4, panel 1). For the results of statistical analyses refer to Table 3.

Since the process of lipid remodelling that promotes the capacitation-dependent increase in membrane fluidity affects differently the functionally different sperm subpopulation present in the experimental system, we represented the GO-induced changes in CDC in Fig. 4, panels 2–6.

Interestingly, the treatment with GO could negatively interfere with the capacitation-dependent increase in membrane fluidity.

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**Table 1**
Size and zeta-potential of GO in water at 38.5 °C obtained by DLS.

| Size (nm) [Polydispersity] | GO 0.5 μg/mL | GO 1 μg/mL | GO 5 μg/mL | GO 10 μg/mL | GO 50 μg/mL |
|---|---|---|---|---|---|
| Size (nm) [Polydispersity] | $800 \pm 30$ [0.236] | $670 \pm 50$ [0.318] | $440 \pm 35$ [0.345] | $540 \pm 10$ [0.249] | $510 \pm 50$ [0.272] |
| Zeta-potential | $-26 \pm 1$ | $-42 \pm 2$ | $-51 \pm 1$ | $-52 \pm 1$ | $-54 \pm 3$ |