GRAPHENE OXIDE INTERACTS WITH SPERM MEMBRANE AND AFFECTS THEIR ACQUISITION OF FERTILIZING ABILITY

Marina Ramal Sanchez[1], Nicola Bernabò[1], Antonella Fontana[2], Romina Zappacosta[2], Valeria Ettorre[2], Giulia Capacchietti[1], Luca Valbonetti[1], Mauro Mattioli[1, 3], Barbara Barboni[1].

1 Faculty of Veterinary Medicine, University of Teramo, Via Renato Balzarini 1, 64100 Teramo, Italy
2 Department of Pharmacy, University "G. D' Annunzio" of Chieti-Pescara, Via dei Vestini, 66100, Chieti, Italy
3 Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Campo Boario, 64100 Teramo, Italy

INTRODUCTION

Graphene oxide (GO), is one of the most widely investigated water-soluble functionalized analogue of graphene, with applications in regenerative medicine and biotechnology. To date, data on toxicity are conflicting, and althought several reports have shown that GO could have some negative effect on human health [1,2,3] and time, dose- and dimension-dependent cytotoxic effects on important cellular models such as erythrocytes and fibroblasts [1], other studies demonstrated no toxicity of GO on fibroblasts [2].

Due to the lack of works evaluating the toxicity of GO on spermatozoa [3,4], and the controversy of others performed in non-capacitated conditions, we carried out an experimental set up to assess the **effects of GO on mature mammalian spermatozoa**. In particular, we studied the interaction between GO and boar sperm membranes during capacitation (i.e. the process that leads the spermatozoa to gain their fertilizing ability).

MATERIALS AND METHODS

- **Semen samples** were collected and processed using a validated protocol and spermatozoa were incubated under capacitated conditions with or without GO during 4 hours
- **Graphene oxide** (GO) was obtained from graphite using a modified Hummers method
- PSA staining and fluorescence microscopy analysis were performed to evaluate the increase of **acrosome reaction** produced by GO on spermatozoa
- DilC12(3) staining and FRAP (fluorescence recovery after photobleaching) analysis with confocal microscopy were carried out to assess changes in **membrane fluidity**
- **IVF** experiments were performed by obtaining healthy oocytes of follicules isolated from boar ovaries and incubating them with healthy boar spermatozoa treated and untreated with GO. Oocytes were stained with HOECHST 3324 to check the success of fertilization

EXPERIMENTS PERFORMED AND RESULTS

- Spermatozoa were co-incubated with different concentrations of GO (50, 10, 5, 1 and 0.5 µg/mL) in a validated in vitro system able to promote capacitation. At 1h intervals (To, T1, T2, T3 and T4) the percentage of spermatozoa showing structural damages in the acrosomes was evaluated using PSA staining and fluorescence microscopy. Higher concentrations of GO (50 and 10 µg/mL) caused and important loss of acrosomes and were classified as toxic (Figure 1) .
- To assess the changes in membrane fluidity caused by GO (5, 1, and 0.5 µg/mL), spermatozoa were cultured with a lipophilic fluorescent stains for labelling membranes DilC12(3) perchlorate (ultra pure) and observed using a confocal microscopy-based Fluorescence Recovery After Photobleaching (FRAP) method (Figure 3). Membrane fluidity of sperm decrease in a GO dose-dependent manner: as higher is the concentration of GO, higher is the membrane fluidity (Figure 4).
- In vitro fertilization (IVF) experiments were performed to verify the effect of GO on fertilizing ability of spermatozoa. After incubating oocytes with spermatozoa co-incubated with GO (5, 1, and 0.5 µg/mL) and non treated sperm, oocytes were stained with HOECHST and evaluated by fluorescence microscopy. Although GO showed to be toxic at high concentrations, and interesting increasing of the IVF rate was observed in oocytes fertilized with spermatozoa treated with a concentration of GO 1 µg/mL (Figure 2).

The induction of acrosome reaction by GO is concentration-dependent

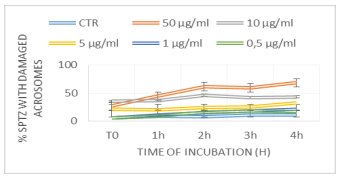


Figure 1. GO induction of acrosome reaction on spermatozoa. Different concentrations of GO were evaluated (50, 10, 5, 1, 0.5 µg/mL) at different capacitation times, confirming a concentration-dependent increase in loss of acrosomes.

GO affects sperm acquisition of fertilizing ability

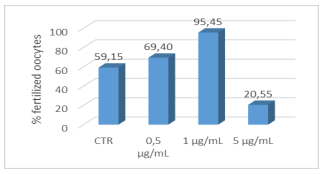


Figure 2. GO effects on IVF experiments. High concentrations of GO were considered as toxic whereas spermatozoa co-incubated with a concentration of 1 µg/mL of GO showed an interesting increase in the number of fertilized oocytes.

GO modifies sperm membrane fluidity

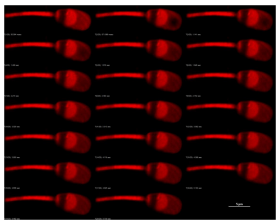
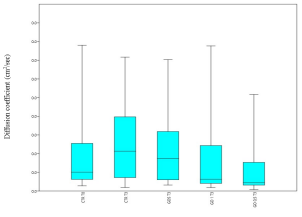


Figure 3. FRAP analysis performed with confocal microscopy. Sequence of one spermatozoa stimulated by photobleaching to calculate the diffusion coeficient.

Figure 4. Fluidity changes on spermatozoa membrane caused by GO. Lower concentrations of GO caused a decrease on spermatozoa membrane fluidity after 3h of capacitation, stabilizing the membrane changes (Exp. performed in 8 different boar models).



CONCLUSIONS

- ✓ Toxic concentrations of GO on spermatozoa those >5 µg/mL
- ✓ GO induces an higher loss of acrosomes on spermatozoa under capacitation conditions
- ✓ GO interacts with the sperm membrane modifying their fluidity in a dose-dependent manner
- ✓ Spermatozoa co-incubated with GO 1 µg/mL produced an increase in the number of fertilized oocytes in vitro

FUTURES PERSPECTIVES

- Spermatozoa adherence to GO: AFM and confocal microscopy
- Study the interactions between graphene and spermatozoa plasma membrane (cholesterol)
- Nanosheets of GO development to use as a diagnostic device

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