Graphene oxide modulates the steroidogenic and apoptotic pathway in a buffalo granulosa cell model

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Background and Rationale

Endocrine Disrupting Chemicals (EDCs) are exogenous substances or mixture that alters functions of endocrine system, causes adverse health effects in an intact organism, its progeny or (sub) populations (WHO, 2002). The dioxins, bisphenol A, persistent organic pollutants (POP), pesticides are among those EDCs. Effects of EDCs cross entire lifespan, with disease burden & costs of US$127 billion annually in Europe & $340 billion in USA (Attina et al., 2016).

The Curious case of Nanomaterials (NMs) !!!

Anthropogenic NMs

Natural NMs (NNMs)	Incidental NMs	Engineered NMs (ENMs)
▪ Forest fires ▪ Volcanic eruptions ▪ Biological molecules (DNA, RNA, Proteins)	• Combustion • vaporization • welding • Grinding…..Byproducts	Intentionally produced with very specific properties or compositions

The engineered nanomaterials (ENMs) are chemicals or materials that are engineered. For example, they can be AuNP, AgNP, Graphene Oxide (GO) among several others. Recent progress in the field of nanotechnology has led to increased exposure to ENMs by animals & humans. However, little is known about the effects of ENMs on reproductive functions. Recent research on ENMs have indicated that they have the potential to perturb the Hypothalamus-Pituitary-Gonadal axis. Graphene Oxide (GO) is one of the ENMs and widely being used and may exert nanotoxicity through ROS. Therefore, we explored the effects of graphene oxide on the steroidogenic and apoptotic pathway in buffalo granulosa cells.

Objectives

The goals of this study are: (1) to understand the effects of GO on genes associated with steroidogenesis in a buffalo granulosa cell model; and (2) to understand the effects of GO on genes associated with apoptotic pathway in a buffalo granulosa cell model; and (3) to understand the effects of GO on exerting oxidative stress and modulation of progesterone hormone in buffalo granulosa cell model

Methodology

1. *In vitro* culture of buffalo ovary Granulosa cells

- Collection of Buffalo ovaries
- Isolation of granulosa cells from small and medium (<8mm) sized follicles
- Cell viability and counting by Trypan blue dye exclusion method
- Granulosa cell culture in 24 well tissue culture plate at a density of 2 x 10^5 viable cells/ml of DMEM

2. Characterization of graphene oxide nanomaterial

- Optical study by measurement of absorption spectra
- Transmission electron microscopy (TEM) and high resolution TEM (HRTEM)
- Study of elemental composition by energy dispersive X-ray spectroscopy (EDS/ EDX)
- Dynamic Light Scattering (DLS) for hydrodynamic diameter and zeta potential

3. Co-incubation of buffalo granulosa cells with graphene oxide and assessing the effects:

Granulosa cells were incubated with graphene oxide (concentration: 2 x 10^9 NP/ml and 2 x 10^{10} NP/ml) for 24 h.

- Assessment of granulosa cell viability by MTT assay
- Gene expression studies for genes of steroidogenesis (β-Hsd, Cyp19A1) & apoptosis pathway (Caspase-3, Bcl-2, Bad, Bax)
- GO effect on Progesterone level during granulosa cell Culture
- GO effect on total antioxidant capacity and lipid peroxidation during granulosa cell culture

Results

1. Characterization of Graphene oxide by TEM, HRTEM, SAED and DLS

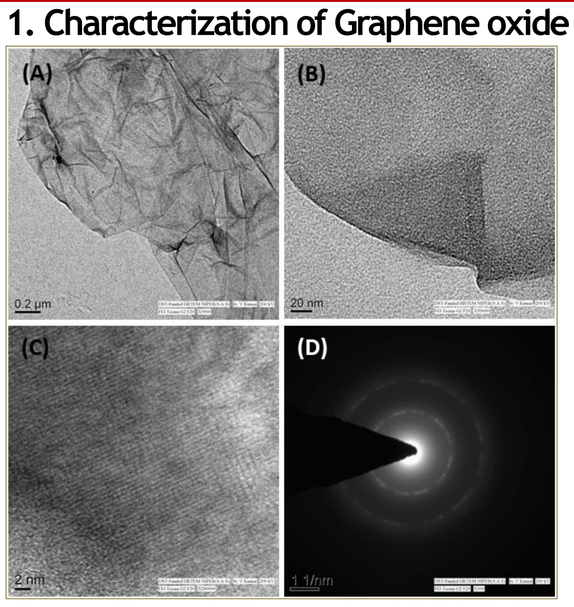

Figure 1: TEM (panel A-B), HRTEM (panel B) & SAED (panel D) images of GO

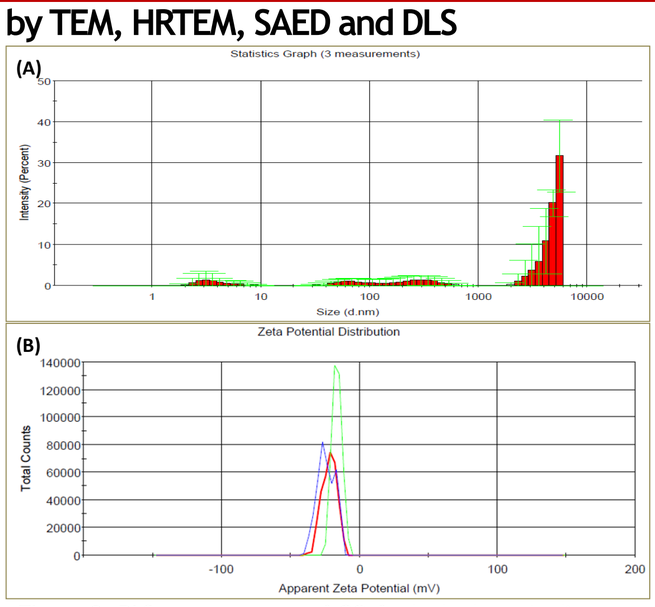

Figure 2: DLS measurements of GO for hydrodynamic diameter (panel A) & zeta potential (panel B)

2. Graphene oxide modulates steroidogenesis in the buffalo granulosa cell model

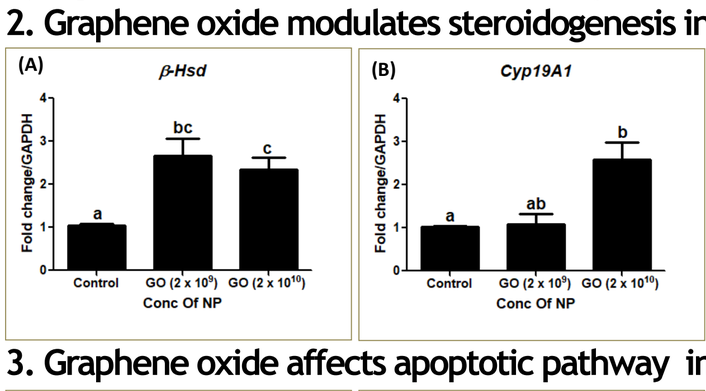

Figure 3: qPCR gene expression results for (A) β-*Hsd* and (B) *Cyp19A1* in granulosa cells treated with graphene oxide (GO). The results are ± S.E.M. of 3 independent experiments. Different letters represents the significant (P<0.05) difference between Control and GO treated cells.

3. Graphene oxide affects apoptotic pathway in the buffalo granulosa cell model

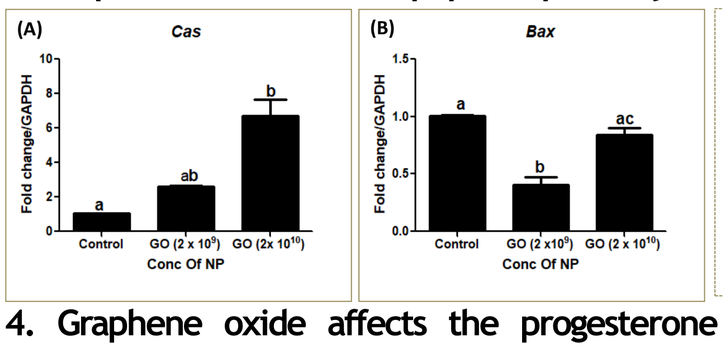

Figure 4: qPCR gene expression results for (A) Caspase-3 and (B) *Bax* in granulosa cells treated with GO. The results are ± S.E.M. of 3 independent experiments. Different letters represents the significant (P<0.05) difference between Control and GO treated cells. No significant changes were found in relative gene expression for Bad & Bcl-2.

4. Graphene oxide affects the progesterone hormone levels during buffalo granulosa cell culture

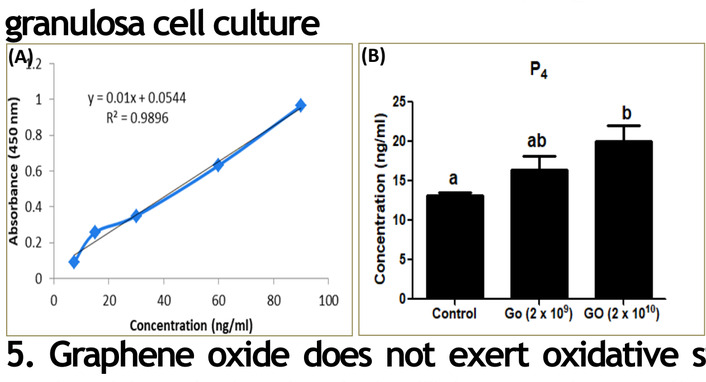

Figure 5: Effect of GO on Progesterone (P_4) levels in buffalo granulosa cells. The results are ± S.E.M. of 3 independent experiments. Different letters represents the significant (P<0.05) difference between Control, GO treated buffalo granulosa cells. (P_4 assay was done by Sigma Bovine Progesterone Assay kit and extrapolation using standard curve with R^2 = 0.9896)

5. Graphene oxide does not exert oxidative stress through lipid peroxidation & anti-oxidant induction in buffalo granulosa cell model

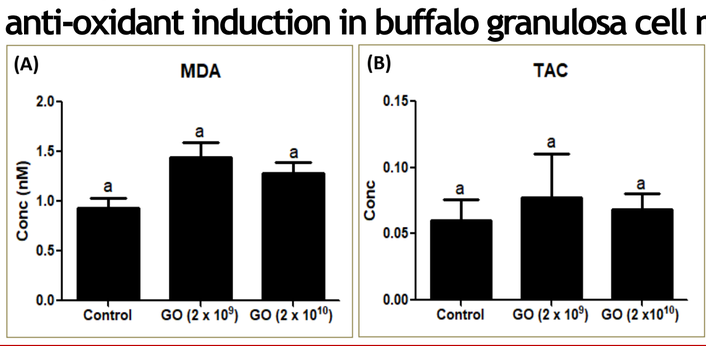

Figure 5: Effect of GO on (A) Lipid peroxidation and (B) Total antioxidant capacity in buffalo granulosa cells. The results are ± S.E.M. of 3 independent experiments. Different letters represents the significant (P<0.05) difference between Control, GO treated buffalo granulosa cells.

Discussion and Conclusion

- GO NPs were characterized by TEM, HRTEM, SAED, DLS etc.
- GO modulates steroidogenesis in the buffalo granulosa cell model by affecting the gene expression of ß-Hsd and Cyp19A1.
- GO affects apoptotic pathway in the buffalo granulosa cell model by affecting the gene expression of Bax and caspase-3.
- Significant changes in progesterone level was found during granulosa cell culture on GO supplementation.
- The GO are not exerting oxidative stress through anti-oxidant induction & lipid Peroxidation in buffalo granulosa cell model.
- The ENMs such as GO may modulate the endocrine system and have the potential to affect the steroidogenic pathway

Future Perspectives

"The rapid growth in Nanotechnology and Engineered Nanomaterials (ENMs) calls into questions about their endocrine modulating abilities, health implications, bioethical and regulatory issues"

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References: (1) WHO, 2002; (2) Attina et al., 2016, Lancet Diabetes Endocrinol.