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Graphene oxide quantum dots disrupt autophagic flux by inhibiting lysosome activity in GC-2 and TM4 cell lines

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ABSTRACT

Graphene oxide quantum dots (GOQDs) have broad application prospects in many areas including bioimaging, drug delivery, DNA cleavage system, sensors and photocatalyst. Recently, increasing concerns have been raised about their biocompatibility, but studies about the effects of GOQDs on male reproductive system are still lacking. In this work, we explored the effects and molecular mechanisms of GOQDs on GC-2 and TM4 cells. We found autophagosome accumulation in GC-2 and TM4 cells after GOQDs treatment. Both LC3-II/LC3-I ratio and p62 levels increased, and the chloroquine-induced accumulation of LC3-II didn't enhance in the presence of GOQDs, which indicated that GOQDs blocked autophagic flux. Further studies found that the fusion between autophagosome and lysosome was not inhibited by GOQDs, but the proteolytic capacity of lysosome was weakened and both the expression and activity of cathepsin B reduced. Taken together, these results suggested that GOQDs blocked autophagic flux by decreasing the amount and enzymatic activity of cathepsin B and inhibiting lysosome proteolytic capacity in GC-2 and TM4 cells, which might have a potential hazard to male reproduction.

1. Introduction

Graphene is composed by single layer of sp^2 carbon atoms with two dimensional honeycomb structure (Novoselov et al., 2004). The unique structure gives it excellent electronic properties such as high intrinsic mobility, good thermal and electrical conductivity. However, because of the zero band gap of graphene, their applications in optoelectronics and photonics are strongly limited (Park and Yan, 2013). To obtain better photoluminescence (PL) property, a variety of graphene derivatives were explored by tuning the size, shape, and fraction of the sp^2 domains in the sp^3 matrix. Among those graphene derivatives, Graphene oxide quantum dots (GOQDs) are considered as rising stars because of their special structures which are similar to graphene, but typically, their lateral diameters are below 100 nm (Liu et al., 2013). Compared to Graphene, GOQDs possess many unique features in terms of better surface grafting, tunable luminescence emission and stable photolumine scence (Li et al., 2013). Owing to their extraordinary luminescent properties, GOQDs can serve as good fluorescent probes for bioimaging (Sun et al., 2013). GOQDs can also be used as vectors to delivery protein or drug molecules to cells because of their high surface area (Wang et al., 2013). Besides, electrochemical biosensor is another application of GOQDs due to their excellent conductivity (Zhou et al., 2015). Therefore, GOQDs are one of the most promising biomaterials for nanomedicine.

Because of such wide applications, the safety of the GOQDs is a natural concern. To date, the biocompatibility of GOQDs has been evaluated by several studies (Chong et al., 2014; Wang et al., 2015). Some researches found that GOQDs had low toxicity *in vivo* (Chong et al., 2014) and *in vitro* (Wu et al., 2013). In contrast, Qin et al. found that GOQDs significantly increased reactive oxygen species generation, apoptotic and autophagic cell death (Qin et al., 2015). Wang et al. (2015) discovered that GOQDs induced the release of reactive oxygen speciesand DNA damage of NIH-3T3 cells. However, studies of their effects on male reproductive system are still lacking.

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Recently, nanoparticles are defined as a novel class of autophagy activators (Zabirnyk et al., 2007), and autophagy dysfunction is an emerging mechanism of nanomaterial toxicity (Stern et al., 2012). Autophagy is a dynamic term for processes by which cytoplasmic materials including organelles are engulfed by double-membrane vesicles and reach lysosomes for degradation (Mizushima et al., 2008). It has been reported that there were autophagy components in spermatocytes (Bustamante-Marin et al., 2012) and autophagy was thought to play an important role in the survival of spermatozoa (Gallardo Bolanos et al., 2012).

GC-2 cell line and TM-4 cell line are derived from immortalized mouse spermatogonia and mouse Sertoli cells, respectively, and they are commonly used for male reproductive toxicity testing (Qu et al., 2014; Xu et al., 2014). Thus, in this study, we aimed to explore the effects of GOQDs on male reproduction using GC-2 and TM4 cells and clarify the molecular mechanisms from the perspective of autophagy.

2. Materials and methods

2.1. Characteristics of GOQDs

GOQDs (product number: XF074) were purchased from XFNANO, INC (Nanjing, China). After sonicated in ice water bath (100 W, 30 min), images of GOQDs were obtained by transmission electron microscope (TEM) (JEOL, Japan). According to the images of TEM, the lateral diameters of about two hundred GOQDs were measured by image J software. The particle size and size distribution of GOQDs in complete cell culture medium were assessed by dynamic light scattering (Brookhaven Instruments Co, USA). Zeta potential was analysed by a Zetasizer Nano series model ZS (Brookhaven Instruments Co, USA).

2.2. Cell culture and co-incubation with GOQDs

GC-2 spd(ts) (ATCC # CRL-2196) and TM4 (ATCC # CRL-1715) cells, purchased from ATCC, were cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, USA). Medium was replaced every day. Before administered to cells, GOQDs were sonicated and diluted to different concentrations (0, 1, 10, 100 μg/mL). The exposure time was 24 h.

2.3. Cell viability assay

Cell viability was analysed using a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). Cells were plated at a density of 1.5×10^4 per well in a 96-well plate (Thermo Scientific, USA) and incubated overnight. After exposure to GOQDs at different concentrations for 24 h, 10 μl CCK-8 solution was added to each well, and then the cells were incubated for 1–4 h at 37 °C in 5% CO_2. The absorbance was determined at 450 nm.

2.4. Cell apoptosis assay

Cell apoptosis was detected by flow cytometry using an Annexin V apoptosis detection kit (BD Biosciences Pharmingen, USA). After GOQDs exposure, cells were trypsinised, washed with PBS and centrifuged at 1000 rpm for 5 min. After washed three times, 1×10^5cells were resuspended in 100 μl of 1 × binding buffer and incubated with 5 μl fluorescein isothiocyanate labeled Annexin V (FITC-Annexin V) and 5 μl propidium iodide (PI) at room temperature avoiding from light for 15 min. Then, 400 μl of 1 × binding buffer was added and the stained cells were collected for flow cytometry analyses.

2.5. Western blotting analysis

Cells were incubated with GOQDs for 24 h, collected and lysed with radio immunoprecipitation assay buffer (RIPA) (Beyotime, China) containing the protease inhibitor cocktail (Sigma, USA). Total protein concentrations were determined by a BCA Protein Assay Kit (Beyotime, China) and each sample was diluted to the same protein concentration. 80 μg of protein was separated by SDS-PAGE and then electroblotted onto PVDF membrane (Bio-Rad, USA). After incubated in blocking buffer for 2 h at room temperature, the membranes were incubated at 4 °C with rabbit anti-LC3 (CST, USA) or rabbit anti-p62 (Abcam, USA) or mouse anti-GAPDH (Beyotime, China) or rabbit anti-Cathepsin B (Santa cruz, USA) overnight. The membranes were washed with Tris-buffered saline with Tween 20 (TBST) and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Beyotime, China) or goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Beyotime, China). Immunoblots were detected by the enhanced chemiluminescence (ECL) western blot detection kit (Millipore, USA) and visualized by Bio-Rad Imaging System.

2.6. Immunofluorescence microscopy

After seeded and incubated in glass bottom dishes overnight, cells were treated with 100 μg/mL GOQDs for 24 h and then fixed with 4% paraformaldehyde for 30 min. Fixed cells were blocked with 1% BSA before incubated with primary antibodies specific for LC3 (CST, USA) or LAMP-2 (abcam, USA) overnight. After washing,

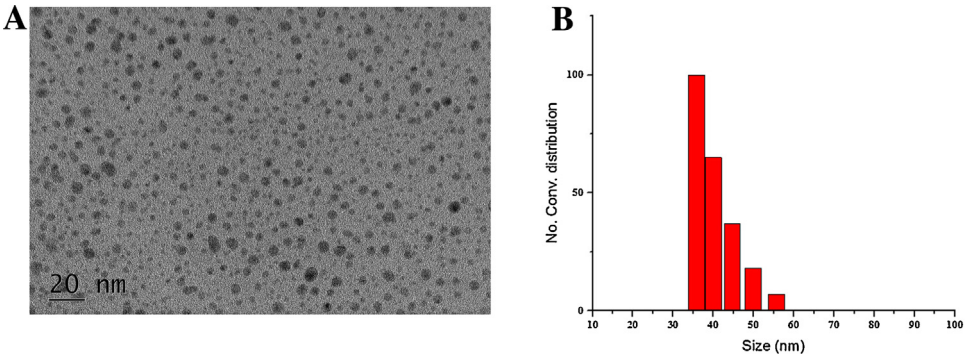

Fig. 1. Characteristics of GOQDs. (A) TEM images of GOQDs. Scale bar = 20 nm. (B) Particle-size distribution of GOQDs prepared in cell complete culture medium.