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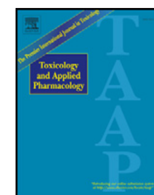

Molecular evidence of offspring liver dysfunction after maternal exposure to zinc oxide nanoparticles

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Recently, reproductive, embryonic and developmental toxicity have been considered as one important sector of nanoparticle (NP) toxicology, with some studies already suggesting varying levels of toxicity and possible transgenerational toxic effects. Even though many studies have investigated the toxic effects of zinc oxide nanoparticles (ZnO NPs), little is known of their impact on overall reproductive outcome and transgenerational effects. Previously we found ZnO NPs caused liver dysfunction in lipid synthesis. This investigation, for the first time, explored the liver dysfunction at the molecular level of gene and protein expression in offspring after maternal exposure to ZnO NPs. Three pathways were investigated: lipid synthesis, growth related factors and cell toxic biomarkers/apoptosis at 5 different time points from embryonic day-18 to postnatal day-20. It was found that the expression of 15, 16, and 16 genes in lipid synthesis, growth related factors and cell toxic biomarkers/apoptosis signalling pathway respectively in F1 animal liver were altered by ZnO NPs compared to $ZnSO_4$. The proteins in these signalling pathways (five in each pathways analyzed) in F1 animal liver were also changed by ZnO NPs compared to $ZnSO_4$. The results suggest that ZnO NPs caused maternal liver defects can also be detected in offspring that might result in problems on offspring liver development, mainly on lipid synthesis, growth, and lesions or apoptosis. Along with others, this study suggests that ZnO NPs may pose reproductive, embryonic and developmental toxicity; therefore, precautions should be taken with regard to human exposure during daily life.

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1. Introduction

Because of their unique properties, nanoparticles (NPs) have been widely used in every aspect of our daily lives. Due to their small size, NPs can pass through biological membranes and into cells (Rothen-Rutishauser et al., 2007) to induce adverse effects on organisms (Wisniewski et al., 2015; Wilson, 1973). Recently, reproductive, embryonic and developmental toxicity have been increasingly considered as one important sector of NP toxicology, as evidenced by the following studies. Maternal exposure to titanium dioxide (Ti_2O) NPs impair brain development and decrease hippocampal cell proliferation in rat offspring (Mohammadipour et al., 2014; Umezawa et al., 2017; Ghaderi et al., 2015) and cause hepatic DNA damage and abnormal gene expression (Jackson et al., 2013). Carbon black (CB) NPs produce liver DNA damage in offspring after maternal inhalation (Jackson et al., 2012) and increase collagen type VIII expression in the kidneys of offspring (Umezawa et al., 2011). Maternal exposure to cadmium oxide nanoparticles induce kidney injury in mice (Blum et al., 2015), and maternal exposure to Cu NPs reduce pup body weight and increase pulmonary inflammation with an increase in the number of neutrophils in BAL fluid compared to controls (Adamcakova-Dodd et al., 2015). Ti_2O exposure during gestation causes abnormal testicular morphology and lowers daily sperm production in mice offspring (Takeda et al., 2009). Gestational exposure to CB NPs adversely affects offspring seminiferous tissue and daily sperm production (Yoshida et al., 2010). In total, these studies suggest that NPs might pose reproductive, embryonic and developmental toxicity with transgenerational toxic effects.

Zinc oxide (ZnO) NPs have also been broadly introduced to human life due to their unique properties such as size, large surface area to volume ratio, typical smoothly scaling properties and others, however, it is also known that ZnO nanomaterials pose potential health risks (Kuang et al., 2016; Filippi et al., 2015; Abbasalipourkabir et al., 2015). ZnO NPs induce oxidative stress in animal livers (Kuang et al., 2016; Yang et al., 2015), adversely affect metabolism and bioenergetics (Filippi et al., 2016), caused liver and renal lesions and reduced sperm quality

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and quantity (Abbasalipourkabir et al., 2015). Maternal exposure to ZnO NPs increase the incidence of abnormalities and decrease offspring body weight (Hong et al., 2014a, 2014b). Exposure to ZnO NPs during gestation reduces the number of born/live pups, decreases pup body weight, increases fetal resorption, and intact NPs are also distributed to the liver and kidneys of pups (Jo et al., 2013). It has been also found that ZnO NPs present in food (Fröhlich and Fröhlich, 2016; Croteau et al., 2011). Due to the small size, ZnO NPs can easily pass through the biological barriers (Zhao et al., 2014). Even though many studies have investigated the toxic effects of ZnO NPs, little is known of their impact on overall reproductive outcome and transgenerational effects.

In our recent study, we found that ZnO NPs decrease egg yolk lipid content in hens, which was correlated with liver dysfunction (Zhao et al., 2016). Because most egg lipids are synthesized in the liver then transferred into egg yolk though the blood, the ability of ZnO NPs to decrease liver lipid synthesis, in turn, decrease egg yolk lipid content. We found that lipid synthesis enzyme gene expression was reduced in the liver, which indicates that liver function might be also disrupted (Zhao et al., 2016). Publications regarding nano-reproductive, embryonic and developmental toxicity to date are largely descriptive in nature regarding the effects of nanoparticles (Poma et al., 2014). Thus, Poma et al. suggest that future investigations should explore interactions between nanomaterials and transgenerational matter on a molecular level (Poma et al., 2014). Since nanoparticles have been found to pose transgenerational effects and ZnO NPs caused liver dysfunction in our previous study (Zhao et al., 2016), the hypothesis of this investigation was that toxic effects of ZnO NPs on hen's liver might pass to the offspring. Therefore, the current investigation aimed to explore the impacts of ZnO NPs on the offspring liver after maternal exposure at a molecular level.

2. Materials and methods

2.1. Characterization of ZnO NPs

ZnO NPs were synthesized by Beijing DK Nano Technology Co. LTD (Beijing, China) as reported in our recent publications (Zhao et al., 2015, 2016). The characteristics of ZnO NPs (morphology, size, agglomeration, etc.) were determined by transmission electron microscopy (TEM; JEM-2100F, JEOL Inc., Japan) and dynamic light scattering (DLS) particle size analyzer (Nano-Zetasizer-HT, Malvern Instruments, Malvern, UK).

2.2. Animal study design (diets and treatments) and sample collection

This investigation was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Qingdao Agricultural University IACUC (Institutional Animal Care and Use Committee) (Zhao et al., 2016). All hens (Jinghong-1 strain) were housed in a ventilated and conventional caged commercial poultry house with a lighting program of 16:8 light/dark and ad-lib food and water. The formulation of the basal diet (corn-soybean base) has been previously reported [Supplementary Table 1 (Table S1)] (Zhao et al., 2016; Liu et al., 2016). The experimental feeding time was from 6 wks to 30 wks of age. The main purpose of this investigation was to explore the different effects on livers from ZnO NPs compared to $ZnSO_4$. In this study, $ZnSO_4$ or ZnO NPs was added to the normal (basal) diet (Table S1) to have two treatment groups: ZnSO4 group, ZnO NPs group. $ZnSO_4$ group was used as control since $ZnSO_4$ is the common diet additive for animals. The concentration of Zn (mg/kg) addition was based on diet. The two treatments were $ZnSO_4$-200 mg/kg and ZnO-NP-200 mg/kg of diet. A total of 400 pullets were randomly assigned to the two treatments, with five replicates per treatment and forty animals per replicate. After 24 wks treatments, the hens were artificially inseminated with fresh, diluted semen 0.03 mL/hen providing about 210 million sperm. Eggs were collected and stored at 13 °C and 75% relative humidity for 5 days until placed in incubators. After hatching, the F1 chickens were raised under same conditions on the same diet (no additional ZnO NPs treatment for F1 animals). Liver samples were collected at embryonic day 18 (E-18), postnatal day 3 (d-3), postnatal day 5 (d-5), postnatal day 10 (d-10) and postnatal day 20 (d-20) and the liver samples were frozen immediately in liquid nitrogen for further analysis (6 animals/group). Part of the liver samples were fixed in 10% neutral formalin and then paraffin embedded. Subsequently, 5-μm sections were prepared and stained with hematoxylin and eosin (H&E) for the analysis of morphological changes (Zhao et al., 2010).

2.3. Real-time quantitative RT-PCR

The procedure for mRNA q-RT-PCR was reported in our early publications (Liu et al., 2016). RNA from liver tissues was extracted using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) and purified using an RT2 qPCR-Grade RNA Isolation Kit from SABiosciences Co., Ltd. (MD, USA). Total RNA was quantified using a Nanodrop 3300 (ThermoScientific, DE, USA). The quality of RNA was controlled by the A260:A280 ratio being >2.0 and confirmed by electrophoreses, with a fraction of each total RNA sample with sharp 18S and 28S ribosomal RNA (rRNA) bands as reported in our recent publication (Liu et al., 2016). One microgram of total RNA was used to make the first strand cDNA in 20 μl. The program for the reaction of miRNA and lncRNA was 25 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, then 4 °C or on ice. The qPCR was performed with the Roche LightCycler 480 (Roche, Germany) and the reaction was as follows: Step 1, 95 °C for 3 min; Step 2, 40 cycles of 95 °C for 12 s; 62 °C for 40 s. The primer sets for mRNA are given in Table S2. Three independent experimental samples were analyzed. q-RT-PCR was statistically analyzed using proprietary software from SABiosciences online support (www.SABiosciences.com).

2.4. Western blotting

Liver samples were lysed in RIPA buffer containing a protease inhibitor cocktail from Sangon Biotech, Ltd. (Shanghai, China). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology, Shanghai, PR China) (Zhao et al., 2015). The information for the primary antibodies (Abs) is present in Table S3. GAPDH and Actin were used as loading controls. Secondary donkey anti-goat Ab (Cat no. A0181) was purchased from Beyotime Institute of Biotechnology, and goat anti-rabbit (Cat no.: A24531) Abs were bought from Novex® by Life Technologies (USA). Fifty micrograms of total protein per sample were loaded onto 10% SDS polyacrylamide electrophoresis gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 2.5 h at 4 °C. Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature (RT), followed by three washes with 0.1% Tween-20 in TBS (TBST). The membranes were incubated with primary Abs diluted at 1:500 in TBST with 1% BSA overnight at 4 °C. After three washes with TBST, the blots were incubated with the HRP-labeled secondary goat anti-rabbit or donkey anti-goat Ab respectively for 1 h at RT. After three washes, the blots were imaged (Zhao et al., 2015). For WB analysis, two livers (from two animals) were mixed to form one sample, and three samples (totally six livers) from each treatment were analyzed at each time point. The WB images were quantified by Image J. The data were normalized to the protein of house-keeping gene (GAPDH) firstly, then the data from ZnO NPs treatment were compared to the data from ZnSO4 treatment.

2.5. Statistical analyses

The data were statistically analyzed using SPSS statistical software (IBM Co., NY, USA) and ANOVA. Comparisons between groups were

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