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Regulation of neuroendocrine cells and neuron factors in the ovary by zinc oxide nanoparticles

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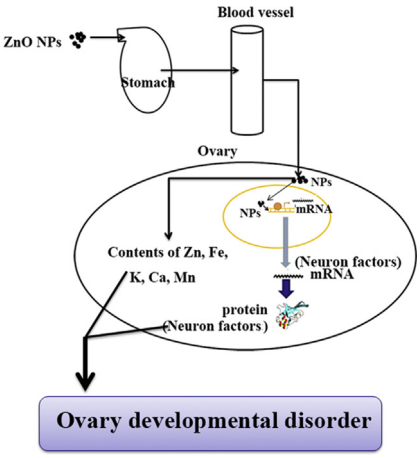
HIGHLIGHTS

- First *in vivo* investigation of the effects of ZnO NPs on neuron factors and neuroendocrine cells.
- ZnO NP altered gene and protein expression of neuron factors in ovary.
- ZnO NPs treatments differentially regulated the population of neuro-endocrine cellsin ovary.
- ZnO NPs also changed the essential elements contents in ovary.
- ZnO NPs might adversely affect neuron systems in ovary.

GRAPHICAL ABSTRACT



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ABSTRACT

The pubertal period is an important window during the development of the female reproductive system. Development of the pubertal ovary, which supplies the oocytes intended for fertilization, requires growth factors, hormones, and neuronal factors. It has been reported that zinc oxide nanoparticles (ZnO NPs) cause cytotoxicity of neuron cells. However, there have been no reports of the effects of ZnO NPs on neuronal factors and neuroendocrine cells in the ovary (*in vivo*). For the first time, this *in vivo* study investigated the effects of ZnO NPs on gene and protein expression of neuronal factors and the population of neuroendocrine cells in ovaries. Intact NPs were detected in ovarian tissue and although ZnO NPs did not alter body weight, they reduced the ovary organ index. Compared to the control or $ZnSO_4$ treatments, ZnO NPs treatments differentially regulated neuronal factor protein and gene expression, and the population of neuroendocrine cells. ZnO NPs changed the contents of essential elements in the ovary; however, they did not alter levels of the steroid hormones estrogen and progesterone. These data

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together suggest that intact ZnO NPs might pose a toxic effect on neuron development in the ovary and eventually negatively affect ovarian developmental at puberty.

1. Introduction

The pubertal period is an important window in the development of the female reproductive system. In 1998, the Food and Drug Administration (FDA) issued the Pediatric Rule requiring the pharmaceutical industry to assess the safety of all new drugs likely to be used for children (Bonnet et al., 2013). Subsequently, the FDA and the European Medicines Agency (EMA) issued guidance documents stating that the reproductive system, among others, is considered at high risk for drug toxicity since this system undergoes significant postnatal developmental changes (Bonnet et al., 2013; Tassinari et al., 2011; US EPA, 2009). The ovaries are integral to the female reproductive system and produce the oocytes intended for fertilization (Picut et al., 2015). Development of the pubertal ovary requires nutrients such as growth factors and hormones (Johnson and Woods, 2009; Onagbesan et al., 2009). Neuronal factors also play important roles in ovarian development (Dees et al., 2006). It has been proposed that mammalian ovaries are innervated by sympathetic and sensory neurons extrinsic to the gland and that they also have intrinsic innervation through neurons residing within the gland (Dees et al., 2006).

Domestic hens (*Gallus gallus*) are important model organisms because they bridge the evolutionary gap between mammals and other vertebrates (International Chicken Genome Sequencing Consortium, 2004). Due to the experimental advantages of *in ovo* embryogenesis, chicken embryos provide a useful vertebrate system for developmental biologists; the reproductive physiology of the domestic hen, especially the ovary, is fascinating and challenging (Bahr and Johnson, 1984). Hofmann et al. (2013) used immunoflourescent technology with neurofilaments, synaptophysin, and chromogranin-A to determine that neuroendocrine cells are present in the domestic hen's ovary. These cells also display immunoreactivity for tyrosine, tryptophan, and dopamine b-hydroxylases, as well as for aromatic L-DOPA Decarboxylase.

Due of their nanoscale properties, zinc oxide nanoparticles (ZnO NPs) have been intensively used in almost all aspects of our life including industrial products (e.g. plastics, ceramics, glass, cement, rubber, paints, and pigments), medical disinfection to inhibit the growth of bacteria (Dwivedi et al., 2014), fungi (He et al., 2011), and viruses (Antoine et al., 2012; Mishra et al., 2011), and sunscreens and cosmetics due to their excellent UV absorption and reflective properties. Most impressively, the small size also helps ZnO NPs to be readily absorbed into biological systems through cellular uptake and the interaction with internal or membrane molecules (Zhao et al., 2014). However, numerous studies report that ZnO NPs cause adverse effects on organisms such as toxicity in *Daphnia magna* (Lopes et al., 2014; Poynton et al., 2011), zebrafish embryos (Brun et al., 2014), rat reproductive development (Jo et al., 2013), mouse spermatogenesis (Talebi et al., 2013), human hepatocyte cells, immune cells, and others (Kim et al., 2013; Tuomela et al., 2013; Papavlassopoulos et al., 2014). It has also been noted that NPs pose neuronal toxicity (Pandey et al., 2015; Valdiglesias et al., 2013; Chiang et al., 2012; Win-Shwe and Fujimaki, 2011); however, these were limited to *in vitro* studies. There are no reports on the effects of ZnO NPs on neuronal factors and neuroendocrine cells in the ovary (*in vivo*). Therefore, the objective of this investigation was to determine the effect of ZnO NPs on the expression of neuronal factors and the population of neuroendocrine cells in the ovary. Domestic hens were chosen as subjects because the hen's ovary is a good model for endocrine studies (Bahr, 1990) and neuroendocrine cells have been clearly identified within it (Hofmann et al., 2013).

2. Materials and methods

2.1. ZnO NP characterization

The methods used for characterizing ZnO NPs are reported in our previous publications (Zhao et al., 2016, 2015). Briefly, ZnO NPs were synthesized by Beijing DK Nano Technology Co. Ltd, Beijing, China. Morphology, size, and agglomeration were characterized 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; Zhao et al., 2016).

2.2. Study design (diets and treatments) and sample collection

Animal experimental procedures have been reported in our recent paper (Zhao et al., 2015); these followed the regulations of the animal ethics committee of Qingdao Agricultural University (Zhao et al., 2016; Zhang et al., 2016). The experiments were performed in a commercial poultry house at Maochangyuan Co., Qiangdao, China. All hens (Jinghong-1 strain) were housed in an enclosed, ventilated, and conventional caged house with a lighting program of 16:8 light/dark cycles and 55% relative humidity with free access to food and water. The composition of the basal diet (corn-soybean base) has been previously reported (Zhao et al., 2016) [Supplemental Table 1 (Table S1)].

To compare the different effects of intact NPs and Zn^{2+}, $ZnSO_4$ was used in this investigation to provide sole Zn^{2+}. In order to determine the effect of NPs on ovarian tissue Zn content, concentration gradients of ZnO NPs and ZnSO4 were used. There were 7 treatments based on the concentration of Zn (mg/kg diet): (1) Control treatment (no Zn added); (2) $ZnSO_4$-25 mg/kg; (3) $ZnSO_4$-50 mg/kg; (4) $ZnSO_4$-100 mg/kg; (5) ZnO-NP-25 mg/kg; (6) ZnO-NP-50 mg/kg; (7) ZnO-NP-100 mg/kg. Zn content of the control diet was quantified to be 12.02 ± 0.31 mg/kg. Pullets ($n = 336$) were randomly assigned to 7 treatments, with 3 replicates per treatment and 16 hens per replicate.

Body weight was recorded at the beginning and end of the experimental period, and feed intake was determined once a week. At 10 weeks of age, 12 hens from each treatment were humanely killed and blood (plasma) and organ samples were collected and stored at $-80\,^{\circ}C$.

2.3. Detection of ZnO NPs in tissues by transmission electron microscopy (TEM) and energy disperse spectroscopy (EDS)

Sample preparation procedures for the detection of NPs took place as previously reported (Zhang et al., 2016; Zhao et al., 2016, 2015). Briefly, tissue samples were collected and fixed for 2 h in 2% glutaraldehyde made in sodium phosphate buffer (pH 7.2). Specimens were then extensively washed to remove excess fixative and subsequently post-fixed in 1% OsO_4 for 1 h in the dark. The specimens were dehydrated in an increasing graded series of ethanol and infiltrated with an increasing concentration of Spur's embedding medium in propylene epoxide. Then the specimens were polymerized in embedding medium for 12 h at 37 °C, 12 h at 45 °C, and 48 h at 60 °C. Fifty nanometer sections were