Toxicology and Applied Pharmacology 329 (2017) 318–325

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

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

Yanan Hao [a,1], Jing Liu [b,1], Yanni Feng [a], Shuai Yu [a], Weidong Zhang [a], Lan Li [a], Lingjiang Min [a], Hongfu Zhang [c], Wei Shen [a], Yong Zhao [a,c,*]

[a] College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, China
[b] Core Laboratories of Qingdao Agricultural University, Qingdao 266109, China
[c] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China

ARTICLE INFO

Article history:
Received 22 April 2017
Revised 8 June 2017
Accepted 21 June 2017
Available online 23 June 2017

Keywords:
ZnO nanoparticles
Offspring
Liver dysfunction
Gene expression
Protein level

ABSTRACT

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.

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

* Corresponding author at: College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, China.
E-mail address: yzhao@msu.edu (Y. Zhao).
[1] Co-first author.

http://dx.doi.org/10.1016/j.taap.2017.06.021
0041-008X/

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

tested by One-Way ANOVA analysis and the LSD test. All the groups were compared with each other for every parameter (mean ± SE). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of ZnO NPs

The ultra-structure of ZnO NPs and their characterization in cells or tissues have been reported [Supplemental Fig. 1 (Fig. S1)] (Zhao et al., 2015, 2016). ZnO NPs used in our studies were morphologically nearly spherical with a milky white colour. The size was approximately 30 nm, surface area was around 50 m^2/g and density was 5.606 g/cm^3 (Fig. S1).

3.2. Histological liver lesions developed in F1 chickens

In our previous reports, 10–200 mg/kg (diet) ZnO NP or $ZnSO_4$ treatments did not alter hen body weight or egg production (Zhao et al., 2016). NPs were found in ZnO NP treated hen ovarian tissues (Fig. S1) (Zhao et al., 2016). ZnO NP or $ZnSO_4$ treatments hardly changed Zn content in ovarian tissues (Fig. S2) (Zhao et al., 2016). However, the ZnO-NP-200 mg/kg treatment decreased lipid content compared to that in the $ZnSO_4$-200 mg/kg treatment (Zhao et al., 2016). In order to retain consistency with our earlier study, in the current investigation, $ZnSO_4$-200 mg/kg and ZnO-NP-200 mg/kg treatments were used as 200 mg/kg is a common inclusion rate in hen diets. ZnO NP or $ZnSO_4$ concentrations used in our studies were calculated on the basis of the diet. If the concentration of 200 mg/kg of diet was calculated based on animal body weight (BW), it would be equivalent to around 20 mg/kg BW. Therefore, our experimental concentration was lower than that used in other studies (200–400 mg/kg BW) (Hong et al., 2014a, 2014b).

In our recent publication, we report that ZnO NPs cause liver dysfunctions with a decrease in lipid synthesis in hen livers (Zhao et al., 2016). The effects of ZnO NPs on light microscopy histopathological liver tissue changes in F1 chickens are shown in Fig. S3. In the $ZnSO_4$-200 mg/kg treatment, hepatocellular cords and the shape of hepatocytes were regular from E-18 to d-20 (Fig. S3). In the ZnO NP treatment, the hepatocellular cords were also regular; however, infiltrated inflammatory cells were observed on d-20, liver hepatocytes had irregular organization with lesions from d-5 and the affected hepatocytes had pyknotic nuclei on d-20 (Fig. S3). Similar phenomena have been seen with F0 hens (data not shown).

3.3. Abnormal gene expression and protein levels of lipid synthesis enzymes due to ZnO NPs in F1 chicken livers

In our previous study (Zhao et al., 2016), we found that ZnO NPs decreased lipid synthesis through a reduction in gene expression of lipid synthesis enzymes in animal livers. In the current investigation, we determined the gene and protein levels of lipid synthesis enzymes. At E-18 and d-3, the gene levels of all lipid synthesis enzymes analyzed (FANS, DECR1, ECI1, ELOVL1, ELOVL2, ELOVL4, ELOVL5, ELOVL6, ELOVL7, GPAM, AGPAT3, Lss, CYP51A1, Nsdh1 and Dhcr7) were down-regulated in the ZnO NP group compared with the $ZnSO_4$ group (Fig. 1). Starting from d-5, gene levels of some of the lipid synthesis enzymes in the ZnO NP group began to increase to similar to, or even higher than those in the $ZnSO_4$ group. At d-10 and d-20, gene levels of most of the lipid synthesis enzymes continued to increase in the ZnO NP group to levels above the $ZnSO_4$ group (Fig. 1).

Five important proteins for lipid synthesis: fatty acid synthase (FASN), nuclear receptor subfamily 1 group H member 3 (NR1H3), acyl CoA synthetase long chain family member 1 (ACSL1), insulin induced gene 1 (INSIG1) and sterol regulatory element binding protein 1 (SREBF1) were altered in F1 chicken livers by ZnO NPs. The protein level of FASN was reduced in the ZnO NP group at E-18 and d-5 as compared to the $ZnSO_4$ group (Fig. 2A&B). However, FASN protein level was elevated in the ZnO NPs group to be higher than that in the $ZnSO_4$ group at d-10 and it was similar between both groups at d-20 (Fig. 2A&B). The protein level of NR1H3 showed a similar trend to FASN protein level at E-18, d-5 and d-10, while it showed a greater decrease in the ZnO NP group than in the $ZnSO_4$ group at d-20. Protein levels of ACSL1, INSIG1 and SREBF1 were similar between ZnO NPs and ZnSO4 at E-18 and d-5, and they were higher in the ZnO NP group than in the $ZnSO_4$ group at d-10 (Fig. 2A&B). However, at d-20, ACSL1 was lower, INSIG1 was higher and SREBF1 was similar to those found in the $ZnSO_4$ group. A similar trend for all five proteins showed them to be higher in the ZnO NP than in the $ZnSO_4$ group at d-10 (Fig. 2A&B). ZnO NPs caused liver lipid synthesis problems in F0 hens and F1 chickens.

3.4. The gene expression and protein levels of growth related factors altered by ZnO NPs in F1 chicken livers

Since the toxic effects of ZnO NPs on liver lipid synthesis were found both in maternal and offspring animals, next we aimed to determine whether the growth-related factors in F1 chicken livers were altered or not. Fifteen growth related factors (HGF, GHR, IGF1R, IGF2, TGFB2, TGFB3, GHRHR, GDF2, GDF3, GDF5, GDF7, GDF8, GDF10, GHRH and IGF2R) were altered in F1 chicken livers in the ZnO NP group compared

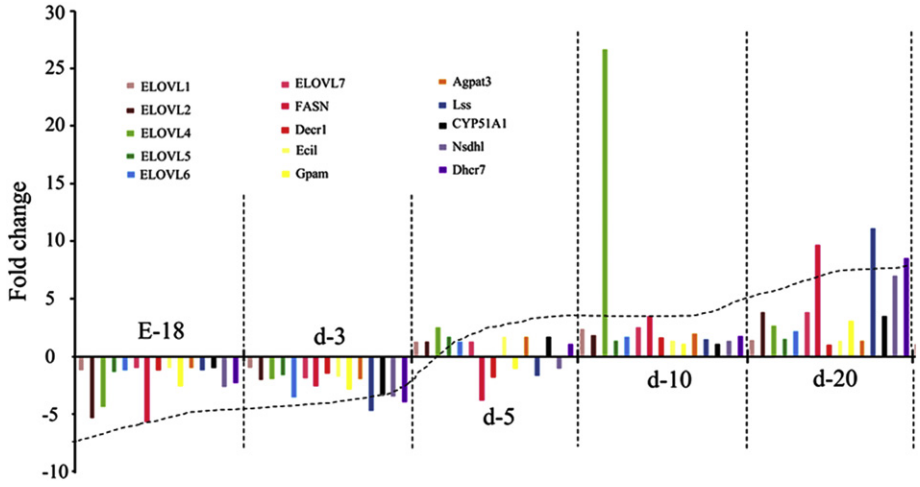

Fig. 1. Abnormal gene expression of lipid synthesis enzymes by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. Six animal samples were analyzed in each treatment at each time point. The data were normalized to house-keeping genes (GAPDH and beta-actin), then the data from ZnO NPs treatment were compared to the data from ZnSO4 treatment.

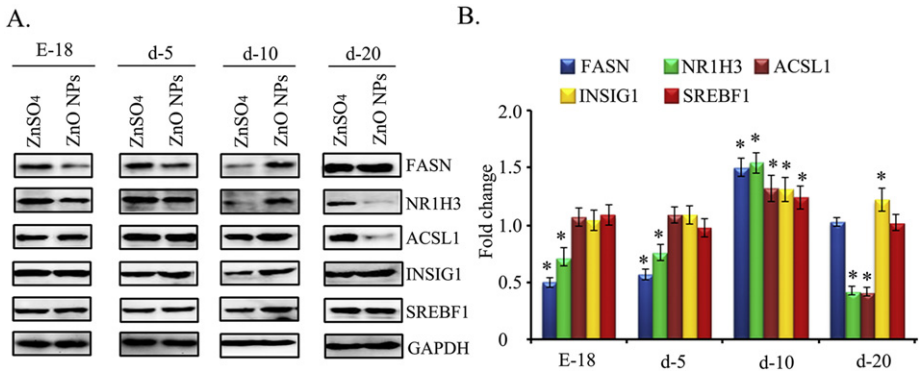

Fig. 2. Abnormal protein levels of lipid synthesis enzymes by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. A. WB images of five lipid synthesis enzymes. B. Quantitative data for WB analysis. For WB analysis, two livers (from two animals) were mixed to form one sample, and three samples (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. $^{*}P < 0.05$.

to the $ZnSO_4$ group. At E-18, all 15 genes were down-regulated in the ZnO NPs group as compared to the ZnSO4 group (Fig. 3). At d-3 and d-5, most of these genes were similar between these two groups and some were higher in the ZnO NP group (Fig. 3). At d-10 and d-20, most of the genes were higher in the ZnO NP group over the $ZnSO_4$ group, and most of them were highest at d-10 (Fig. 3).

Five proteins related to cell growth: proliferating cell nuclear antigen (PCNA), phosphatidylinositol 3 kinase (PI3K), insulin like growth factor 1 receptor (IGF1R), phosphor-extracellular signal regulated kinase 1 (p-ERK) and phosphor-protein kinase B (p-AKT) were changed in F1 chicken liver samples by ZnO NPs (Fig. 4A&B). PCNA was lower in the ZnO NP group than in the $ZnSO_4$ group at E-18; however, it was higher in the ZnO NP group at d-20. Furthermore, at d-5 and d-10 it was similar between the two groups. The protein level of PI3K was higher in the ZnO NP group than in the $ZnSO_4$ group at d-20, but it was lower than the latter at d-10, while the groups were similar at E-18 and d-5. The protein level of IGF1R was lower in the ZnO NP group at E-18, d-10 and d-20 while groups were similar at d-5. The protein level of p-ERK was lower in the ZnO NP group at E-18 while the two groups were similar at other time points. The protein level of p-AKT was lower in the ZnO NP group at E-18, higher at d-20 and similar to that in the $ZnSO_4$ group at d-5 and d-10. ZnO NPs caused toxic effects on cell growth in F1 chicken liver.

3.5. Cell damage or apoptosis induced in the ZnO NP group F1 chicken livers

The gene levels of sixteen factors (MIF, CHOP, IGF-1, SIRT1, AKIP1, IAP, Mff, Apaf-1, TFAM, OCT, SIRT3, HERPUD2, XBP-1, TRIB3, eIF2a and HSP70) related to mitochondrial or endoplasmic reticulum (ER) stress induced damage markers were altered in F1 chicken livers by ZnO NPs. At E-18 the gene expression of IGF-1 and TRIB3 were significantly lower in the ZnO NP group than in the $ZnSO_4$ group, while they were highest at d-10. At d-5, most of the levels of these genes were lower in the ZnO NP group; however, at d-10 almost all gene levels were higher in the ZnO NP group. At d-20, some gene levels were higher and some were lower in the ZnO NP group as compared to the $ZnSO_4$ group (Fig. 5).

Five proteins (CHOP, TRIB3, p53, capase3 and caspase8) related to cell damage or apoptosis were altered in F1 chicken liver by ZnO NPs. Protein levels of TRIB3 correlated well with its gene expression level; it was lower in the ZnO NP group at E-18, but was higher in the same group at d-5, d-10 and d-20. The protein level of CHOP remained similar

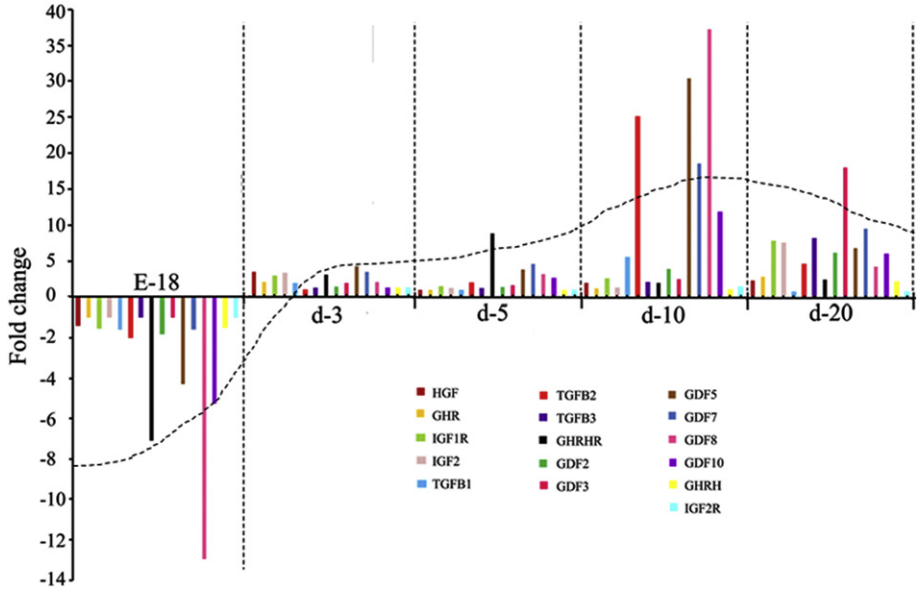

Fig. 3. Abnormal gene expression of growth related factors by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. Six animal samples were analyzed in each treatment at each time point. The data were normalized to house-keeping genes (GAPDH and beta-actin), then the data from ZnO NPs treatment were compared to the data from ZnSO4 treatment.

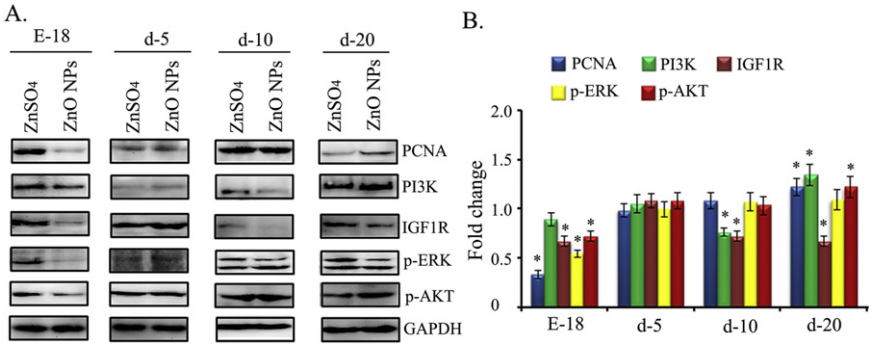

Fig. 4. Abnormal gene expression and protein levels of growth related factors by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. A. WB images of five growth related factors. B. Quantitative data for WB analysis. For WB analysis, two livers (from two animals) were mixed to form one sample, 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. *$P < 0.05$.

in both groups at E-18, d-5 and d-10, however, it was higher in the ZnO NP group at d-20. The protein level of p53 was higher in the ZnO NP group at E-18 and d-20, while it was similar in the two groups at d-5 and d-10. Protein levels of caspase 3 and caspase 8 were higher in the ZnO NP group at d-5, d-10 and d-20 than in the $ZnSO_4$ group, while they were similar in these two groups at E-18. The data suggest that apoptosis or cell damage may develop in F1 chicken livers in the ZnO NP group (Fig. 6A&B).

4. Discussion

ZnO NPs are commonly used in sunscreen, cosmetics, biocides and in many aspects of our daily lives (Bondarenko et al., 2013; Ma et al., 2013; Cho et al., 2013; Brun et al., 2014; Chen et al., 2014a). Although some reports suggest that ZnO NPs pose toxic effects on reproductive systems (Jo et al., 2013) and embryonic growth and development (Lacave et al., 2016; Bonfanti et al., 2015; Brun et al., 2014; Chen et al., 2014a; Hua et al., 2014; Ong et al., 2014; Manzo et al., 2013; Zhao et al., 2013; Xia et al., 2011), little is known about their impacts and underlying mechanisms on the offspring development, especially at a molecular level. Our recent investigation found ZnO NPs caused liver problems through decreasing liver lipid synthesis (Zhao et al., 2016). The metabolism of lipids in most animals includes three aspects: (1) predominant synthesis in the liver, (2) transportation and degradation in blood and (3) absorption, usage and storage in tissues (Huang and Freter, 2015; Athenstaedt and Daum, 2006; Attia et al., 2014). Lipid synthesis is a long process employing different enzymes and is also regulated by dietary and husbandry factors (Huang and Freter, 2015; Athenstaedt and Daum, 2006; Attia et al., 2014). The expression of major genes for lipid synthesis was reduced by ZnO-NP-200 mg/kg in hen's livers after 24 weeks of treatment (30 weeks of age).

In the current investigation, hens were on ZnO NP or $ZnSO_4$ diets for 24 weeks (30 weeks of age) then, after fertilization and hatching, liver samples were collected from the offspring at 5 different time points from E-18 to 20-d. Three major pathways involved in lipid synthesis, growth related signalling and cell damage or apoptosis were investigated both at gene and protein levels. The results were very interesting. The trend for the expression of the 15 lipid synthesis genes (FANS, DECR1,

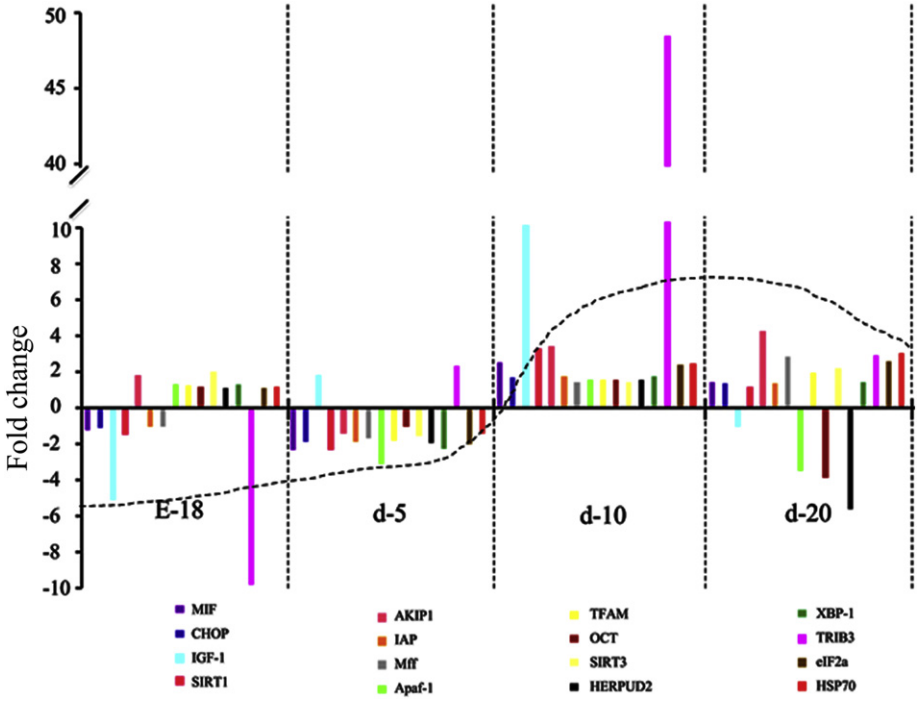

Fig. 5. Gene expression of cell damage biomarkers and apoptosis factors by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. Six animal samples were analyzed in each treatment at each time point. The data were normalized to house-keeping genes (GAPDH and beta-actin), then the data from ZnO NPs treatment were compared to the data from ZnSO4 treatment.

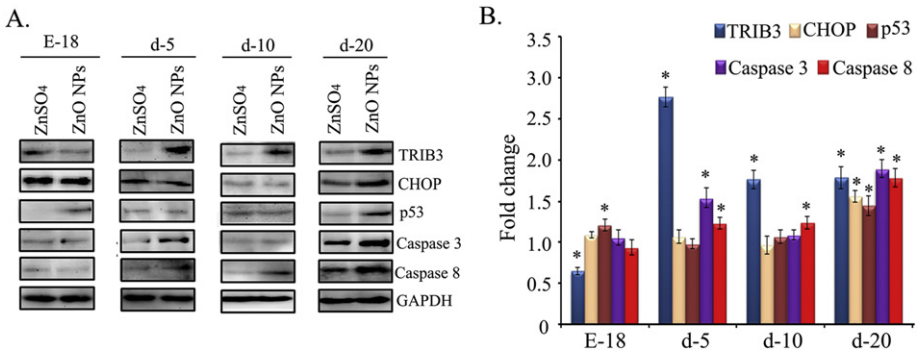

Fig. 6. Protein levels of cell damage biomarkers and apoptosis factors by ZnO NPs in F1 chicken liver at E-18, d-3, d-5, d-10 and d-20. A. WB images of cell damage biomarkers and apoptosis factors. B. Quantitative data for WB analysis. For WB analysis, two livers (from two animals) were mixed to form 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. $^{*}P < 0.05$.

ECI1, ELOVL1, ELOVL2, ELOVL4, ELOVL5, ELOVL6, ELOVL7, GPAM, AGPAT3, Lss, CYP51A1, Nsdh1 and Dhcr7) was down-regulation in ZnO NP groups at early time points (E-18 and d-3), then an increase started from d-5 and reached a peak at d-10 or d-20. Five proteins related to lipid synthesis (FASN, NR1H3, ACSL1, INSIG1 and SREBF1) were also determined in this study. FASN is one of the de novo lipogenic enzymes (Ramírez-López et al., 2016) which play a vital role in liver lipid metabolism. The acyl-CoA synthetase long-chain family member 1 (ACSL1) plays a key role in fatty acid synthesis by catalysing the ATP-dependent acylation of fatty acids into long-chain acyl CoA (Singh et al., 2016; Lian et al., 2016; Joseph et al., 2015). INSIG1, a membrane-spanning (polytopic) protein and a liver-specific cholesterol biosynthesis regulator, regulates lipid synthesis by retaining SREBFs in the ER and preventing their proteolytic activation in the Golgi apparatus (Ramírez-López et al., 2016; Wang et al., 2017). NR1H3, a member of the LXR nuclear super receptor family, is mainly localized in the liver, adipose tissue, kidney, small intestine, and macrophages to maintain homeostasis of cholesterol levels, lipoprotein metabolism and fat synthesis (Shi et al., 2016; Zhang et al., 2016). The trend for protein levels of these five enzymes was similar with highest levels at d-10; however, the levels of FASN, NR1H3 and ACSL1 were more profoundly influenced with a dramatic reduction at E-18, d-5 or d-20. The results in this lipid synthesis pathway indicated that it was altered in the offspring liver by ZnO NP treatment. Data matched well with our early findings that ZnO NPs decreased lipid synthesis enzyme gene expression (Zhao et al., 2016) and other findings that ZnO NPs affected metabolism and bioenergetics in hepatocytes (Filippi et al., 2015). This suggests that ZnO NPs could produce toxic effects on offspring liver through maternal exposure.

Since lipid synthesis function in F1 animal liver was disrupted by ZnO NPs, the growth of the liver might also be altered. The trend for the expression levels of growth related genes were different to that for lipid synthesis enzymes. Five family genes have been analyzed including HGF, GH, IGF, GDF and TGF families (HGF, GHR, IGF1R, IGF2, TGFB2, TGFB3, GHRHR, GDF2, GDF3, GDF5, GDF7, GDF8, GDF10, GHRH and IGF2R). These genes were down-regulated in the ZnO NP group at E-18, then they were increased starting from d-3 and reached their peak at d-10; at d-20 they were lower than at d-10. The trend for protein levels of PCNA, PI3K, IGF1R, p-ERK and p-AKT was similar with an increase with age in F1 animals except for PI3K or IGF1R at d-10 or d-20. The results in this section agree with many early studies that ZnO NPs inhibit growth of organisms (Hong et al., 2014a, 2014b).

It has been assumed that the toxic effects of NPs are due to oxidative stress through endoplasmic reticulum (ER) stress (Kuang et al., 2016; Yang et al., 2015; Chen et al., 2014b). Oxidative stress on the ER could induce ROS production to damage cell membranes. Next we would like to explore whether the ER and mitochondrial stress, and the cell apoptosis pathway in F1 animal livers were also affected. The trend for gene expression of the 16 ER or mitochondrial stress biomarkers was similar to the trend for growth factor gene expression. However, two of these factors, CHOP and IGF-1, were different in that they were down-regulated dramatically at E-18, and up-regulated at d-10 with the highest levels of the 16 biomarkers. This suggested that they might be sensitive biomarkers for ZnO NP induced toxicity. These data agree with an earlier study (Shi et al., 2016). CHOP (also known as DNA-damage-inducible transcript 3, DDIT3) is involved in ER stress related apoptosis (Chen et al., 2014b). The protein level of CHOP was increased at d-20. Another biomarker, TRIB3, was increased at d-5, d-10 and d-20. TRIB3 is a mammalian homolog of Drosophila tribbles and is also considered as a neuronal cell death-inducible putative protein kinase (Qian et al., 2008). The protein levels of p53, caspase 3 and caspase 8 were elevated as the animals aged. These results matched well with histopathological morphological changes in the liver that the lesions developed as the animals became older.

The findings of the present study illustrated that ZnO NPs disrupted three important signalling pathways in liver which might subsequently reduce liver functions. The lipid synthesis genes were altered which may result in systemic shortage of lipid or hepatic steatosis. The liver growth factors were also changed by ZnO NPs which might cause the animal growth retardation. Indeed it was found the body weight (F1 animals) in ZnO NPs group was less compared to ZnSO4 group at 6 month of age (unpublished data). The expression of the apoptosis markers or cell damage markers were increased in the F1 animal liver which correlated with lesions formed in these animal livers. The central role of liver is for detoxification, metabolism and excretion of drugs and xenobiotics and ZnO NPs caused liver dysfunction not only in F0 animals but also in F1 animals, this raises the concerns that ZnO NPs might pose adverse effects on humans due to the broad applications.

In conclusion, this investigation, for the first time, explored the impacts of ZnO NPs on offspring liver function at the molecular level of gene and protein expression after maternal oral exposure. Three pathways have been investigated: lipid synthesis, growth related factors and cell toxic biomarkers/apoptosis at 5 different time points from E-18 to d-20. The results suggest that ZnO NPs could be toxic on offspring liver development, mainly influencing 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 in exposing humans to ZnO NPs in daily life.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2017.06.021.

Funding

This work was supported by the Program for Natural Science Foundation of Shandong Province in China (ZR2015CL045), National Natural

Science Foundation of China (31302166), and Open Project Program of the Zhejiang Key Laboratory of Exploitation and Preservation of Coastal Bio-resource (J2013004).

Conflicts interests

The authors declare no competing financial interest.

Authors' contributions

YZ, YH and JL provided key intellectual input in the conception and design of these studies and YZ wrote the manuscript. YH, JL and SY performed animal experiments. WZ and LL did the western blotting. LM and WS performed the PCR experiments. HZ contributed to the writing of the manuscript. All authors reviewed the manuscript.

Acknowledgment

The TEM and EDS work in this manuscript made use of the resources of the Beijing National Center for Electron Microscopy at Tsinghua University. We would like to thank Ms. Hui-Hua Zhou in Tsinghua University for helping on TEM and EDS analysis.

References

Abbasalipourkabir, R., Moradi, H., Zarei, S., Asadi, S., Salehzadeh, A., Ghafourikhosroshahi, A., Mortazavi, M., Ziamajidi, N., 2015. Toxicity of zinc oxide nanoparticles on adult male Wistar rats. Food Chem. Toxicol. 84, 154–160.

Adamcakova-Dodd, A., Monick, M.M., Powers, L.S., Gibson-Corley, K.N., Thorne, P.S., 2015. Effects of prenatal inhalation exposure to copper nanoparticles on murine dams and offspring. Part Fibre Toxicol. 12, 30.

Athenstaedt, K., Daum, G., 2006. The life cycle of neutral lipids: synthesis, storage and degradation. Cell. Mol. Life Sci. 63, 1355–1369.

Attia, Y.A., Al-Harthi, M.A., Shiboob, M.M., 2014. Evaluation of quality and nutrient contents of table eggs from different sources in the retail market. Ital. J. Anim. Sci. 13, 369.

Blum, J.L., Edwards, J.R., Prozialeck, W.C., Xiong, J.Q., Zelikoff, J.T., 2015. Effects of maternal exposure to cadmium oxide nanoparticles during pregnancy on maternal and offspring kidney injury markers using a murine model. J. Toxicol. Environ. Health A 78 (12), 711–724.

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., Kahru, A., 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Arch. Toxicol. 87 (7), 1181–1200.

Bonfanti, P., Moschini, E., Saibene, M., Bacchetta, R., Rettighieri, L., Calabri, L., Colombo, A., Mantecca, P., 2015. Do nanoparticle physico-chemical properties and developmental exposure window influence nano ZnO embryotoxicity in *Xenopus laevis*? Int. J. Environ. Res. Public Health 12 (8), 8828–8848.

Brun, N.R., Lenz, M., Wehrli, B., Fent, K., 2014. Comparative effects of zinc oxide nanoparticles and dissolved zinc on zebrafish embryos and eleuthero-embryos: importance of zinc ions. Sci. Total Environ. 476–477, 657–666.

Chen, T., Lin, C., Meng, P., 2014a. Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (*Danio rerio*). J. Hazard. Mater. 277, 134–140.

Chen, R., Huo, L., Shi, X., Bai, R., Zhang, Z., Zhao, Y., Chang, Y., Chen, C., 2014b. Endoplasmic reticulum stress induced by zinc oxide nanoparticles is an earlier biomarker for nanotoxicological evaluation. ACS Nano 8 (3), 2562–2574.

Cho, W.S., Kang, B., Lee, J.K., Jeong, J., Che, J.H., Seok, S.H., 2013. Comparative absorption, distribution and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. Part Fibre Toxicol. 10, 9.

Croteau, M.N., Dybowska, A.D., Luoma, S.N., Valsami-Jones, E., 2011. A novel approach reveals that zinc oxide nanoparticles are bioavailable and toxic after dietary exposures. Nanotoxicology 5, 79–90.

Filippi, C., Pryde, A., Cowan, P., Lee, T., Hayes, P., Donaldson, K., Plevris, J., Stone, V., 2015. Toxicology of ZnO and TiO_2 nanoparticles on hepatocytes: impact on metabolism and bioenergetics. Nanotoxicology 9 (1), 126–134.

Fröhlich, E.E., Fröhlich, E., 2016. Cytotoxicity of nanoparticles contained in food on intestinal cells and the gut microbiota. Int. J. Mol. Sci. 17 (4), 509.

Ghaderi, S., Tabatabaei, S.R., Varzi, H.N., Rashno, M., 2015. Induced adverse effects of prenatal exposure to silver nanoparticles on neurobehavioral development of offspring of mice. J. Toxicol. Sci. 40 (2), 263–275.

Hong, J.S., Park, M.K., Kim, M.S., Lim, J.H., Park, G.J., Maeng, E.H., Shin, J.H., Kim, M.K., Jeong, J., Park, J.A., Kim, J.C., Shin, H.C., 2014a. Prenatal development toxicity study of zinc oxide nanoparticles in rats. Int. J. Nanomedicine 9 (Suppl. 2), 159–171.

Hong, J.S., ark, M.K., Kim, M.S., Lim, J.H., Park, G.J., Maeng, E.H., Shin, J.H., Kim, M.K., Jeong, J., Park, J.A., Kim, J.C., Shin, H.C., 2014b. Effect of zinc oxide nanoparticles on dams and embryo-fetal development in rats. Int. J. Nanomedicine 9 (Suppl. 2), 145–157.

Hua, J., Vijver, M.G., Richardson, M.K., Ahmad, F., Peijnenburg, W.J., 2014. Particle-specific toxic effects of differently shaped zinc oxide nanoparticles to zebrafish embryos (*Danio rerio*). Environ. Toxicol. Chem. 33 (12), 2859–2868.

Huang, C., Freter, C., 2015. Lipid metabolism, apoptosis and cancer therapy. Int. J. Mol. Sci. 16, 924–949.

Jackson, P., Hougaard, K.S., Boisen, A.M., Jacobsen, N.R., Jensen, K.A., Møller, P., Brunborg, G., Gutzkow, K.B., Andersen, O., Loft, S., Vogel, U., Wallin, H., 2012. Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: effects on liver DNA strand breaks in dams and offspring. Nanotoxicology 6 (5), 486–500.

Jackson, P., Halappanavar, S., Hougaard, K.S., Williams, A., Madsen, A.M., Lamson, J.S., Andersen, O., Yauk, C., Wallin, H., Vogel, U., 2013. Maternal inhalation of surface-coated nanosized titanium dioxide (UV-titan) in C57BL/6 mice: effects in prenatally exposed offspring on hepatic DNA damage and gene expression. Nanotoxicology 7 (1), 85–96.

Jo, E., Seo, G., Kwon, J.T., Lee, M., Lee, B., Eom, I., Kim, P., Choi, K., 2013. Exposure to zinc oxide nanoparticles affects reproductive development and biodistribution in offspring rats. J. Toxicol. Sci. 38 (4), 525–530.

Joseph, R., Poschmann, J., Sukarieh, R., Too, P.G., Julien, S.G., Xu, F., Teh, A.L., Holbrook, J.D., Ng, K.L., Chong, Y.S., Gluckman, P.D., Prabhakar, S., Stünkel, W., 2015. ACSL1 is associated with fetal programming of insulin sensitivity and cellular lipid content. Mol. Endocrinol. 29 (6), 909–920.

Kuang, H., Yang, P., Yang, L., Aguilar, Z.P., Xu, H., 2016. Size dependent effect of ZnO nanoparticles on endoplasmic reticulum stress signaling pathway in murine liver. J. Hazard. Mater. 317, 119–126.

Lacave, J.M., Retuerto, A., Vicario-Parés, U., Gilliland, D., Oron, M., Cajaraville, M.P., Orbea, A., 2016. Effects of metal-bearing nanoparticles (Ag, Au, CdS, ZnO, SiO_2) on developing zebrafish embryos. Nanotechnology 27 (32), 325102.

Lian, S., Guo, J.R., Nan, X.M., Ma, L., Loor, J.J., Bu, D.P., 2016. MicroRNA Bta-miR-181a regulates the biosynthesis of bovine milk fat by targeting ACSL1. J. Dairy Sci. 99 (5), 3916–3924.

Liu, X., Zhang, H., Zhang, W., Zhang, P., Hao, Y., Song, R., Li, L., Feng, Y., Hao, Z., Shen, W., Min, L., Yang, H., Zhao, Y., 2016. Regulation of neuroendocrine cells and neuron-factors in ovary by zinc oxide nanoparticles. Toxicol. Lett. 256, 19–32.

Ma, H., Williams, P.L., Diamond, S.A., 2013. Ecotoxicity of manufactured ZnO nanoparticles – a review. Environ. Pollut. 172, 76–85.

Manzo, S., Miglietta, M.L., Rametta, G., Buono, S., Di Francia, G., 2013. Embryotoxicity and spermiotoxicity of nanosized ZnO for Mediterranean sea urchin *Paracentrotus lividus*. J. Hazard. Mater. 254–255, 1–9.

Mohammadipour, A., Fazel, A., Haghir, H., Motejaded, F., Rafatpanah, H., Zabihi, H., Hosseini, M., Bideskan, A.E., 2014. Maternal exposure to titanium dioxide nanoparticles during pregnancy; impaired memory and decreased hippocampal cell proliferation in rat offspring. Environ. Toxicol. Pharmacol. 37 (2), 617–625.

Ong, K.J., Zhao, X., Thistle, M.E., Maccormack, T.J., Clark, R.J., Ma, G., Martinez-Rubi, Y., Simard, B., Loo, J.S., Veinot, J.G., Goss, G.G., 2014. Mechanistic insights into the effect of nanoparticles on zebrafish hatch. Nanotoxicology 8 (3), 295–304.

Poma, A., Colafarina, S., Fontecchio, G., Chichiriccò, G., 2014. Transgenerational effects of NMs. Adv. Exp. Med. Biol. 811, 235–254.

Qian, B., Wang, H., Men, X., Zhang, W., Cai, H., Xu, S., Xu, Y., Ye, L., Wollheim, C.B., Lou, J., 2008. TRIB3 [corrected] is implicated in glucotoxicity- and endoplasmic reticulum-stress-induced [corrected] beta-cell apoptosis. J. Endocrinol. 199 (3), 407–416.

Ramírez-López, M.T., Arco, R., Decara, J., Vázquez, M., Rivera, P., Blanco, R.N., Alén, F., Gómez de Heras, R., Suárez, J., Rodríguez de Fonseca, F., 2016. Long-term effects of prenatal exposure to undernutrition on cannabinoid receptor-related behaviors: sex and tissue-specific alterations in the mRNA expression of cannabinoid receptors and lipid metabolic regulators. Front. Behav. Neurosci. 10, 241.

Rothen-Rutishauser, B., Schürch, S., Gehr, P., 2007. Interaction of particles with membrane. In: Donaldson, K., Borm, P. (Eds.), Particle Toxicology. CRC Press, Boca Raton, pp. 139–160.

Shi, H., Zhao, W., Zhang, C., Shahzad, K., Luo, J., Loor, J.J., 2016. Transcriptome-wide analysis reveals the role of PPARγ controlling the lipid metabolism in goat mammary epithelial cells. PPAR Res. 2016, 9195680.

Singh, A.B., Kan, C.F., Dong, B., Liu, J., 2016. SREBP2 activation induces hepatic long-chain acyl-CoA synthetase 1 (ACSL1) expression in vivo and in vitro through a sterol regulatory element (SRE) motif of the ACSL1 C-promoter. J. Biol. Chem. 291 (10), 5373–5384.

Takeda, K., Suzuki, K., Ishihara, A., Kubo-Irie, M., Fujimoto, R., Tabata, M., Oshio, S., Nihei, Y., Ihara, T., Sugamata, M., 2009. Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. J. Health Sci. 55, 95–102.

Umezawa, M., Kudo, S., Yanagita, S., Shinkai, Y., Niki, R., Oyabu, T., Takeda, K., Ihara, T., Sugamata, M., 2011. Maternal exposure to carbon black nanoparticle increases collagen type VIII expression in the kidney of offspring. J. Toxicol. Sci. 36 (4), 461–468.

Umezawa, M., Onoda, A., Takeda, K., 2017. Developmental toxicity of nanoparticles on the brain. Yakugaku Zasshi 137 (1), 73–78.

Wang, H., Shi, H., Luo, J., Yi, Y., Yao, D., Zhang, X., Ma, G., Loor, J.J., 2017. MiR-145 regulates lipogenesis in goat mammary cells via targeting INSIG1 and epigenetic regulation of lipid-related genes. J. Cell. Physiol. 232 (5), 1030–1040.

Wilson, J.G., 1973. Environment and Birth Defects. Academic Press, New York.

Wisniewski, P., Romano, R.M., Kizys, M.M., Oliveira, K.C., Kasamatsu, T., Giannocco, G., Chiamolera, M.I., Dias-da-Silva, M.R., Romano, M.A., 2015. Adult exposure to bisphenol A (BPA) in Wistar rats reducessperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology 329, 1–9.

Xia, T., Zhao, Y., Sager, T., George, S., Pokhrel, S., Li, N., Schoenfeld, D., Meng, H., Lin, S., Wang, X., Wang, M., Ji, Z., Zink, J.I., Mädler, L., Castranova, V., Lin, S., Nel, A.E., 2011. Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in rodent lung and zebra fish embryos. ACS Nano 5 (2), 1223–1235.

Yang, X., Shao, H., Liu, W., Gu, W., Shu, X., Mo, Y., Chen, X., Zhang, Q., Jiang, M., 2015. Endoplasmic reticulum stress and oxidative stress are involved in ZnO nanoparticle-induced hepatotoxicity. Toxicol. Lett. 234 (1), 40–49.

Yoshida, S., Hiyoshi, K., Oshio, S., Takano, H., Takeda, K., Ichinose, T., 2010. Effects of fetal exposure to carbon nanoparticles on reproductive function in male offspring. Fertil. Steril. 93, 1695–1699.

Zhang, B., Shang, P., Qiangba, Y., Xu, A., Wang, Z., Zhang, H., 2016. The association of NR1H3 gene with lipid deposition in the pig. Lipids Health Dis. 15, 99.
Zhao, Y., Tan, Y.S., Haslam, S.Z., Yang, C., 2010. Perfluorooctanoic acid effects on steroid hormone and growth factor levels mediate stimulation of peripubertal mammary gland development in C57BL/6 mice. Toxicol. Sci. 115 (1), 214–224.
Zhao, X., Wang, S., Wu, Y., You, H., Lv, L., 2013. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. Aquat. Toxicol. 136–137, 49–59.
Zhao, C.Y., Tan, S.X., Xiao, X.Y., Qiu, X.S., Pan, J.Q., Tang, Z.X., 2014. Effects of dietary zinc oxide nanoparticles on growth performance and antioxidative status in broilers. Biol. Trace Elem. Res. 160, 361–367.
Zhao, Y., Li, L., Zhang, P.F., Shen, W., Liu, J., Yang, F.F., Liu, H.B., Hao, Z.H., 2015. Differential regulation of gene and protein expression by zinc oxide nanoparticles in hen's ovarian granulosa cells: specific roles of nanoparticles. PLoS One 10 (10), e0140499.
Zhao, Y., Li, L., Zhang, P.F., Liu, X.Q., Zhang, W.D., Ding, Z.P., Wang, S.W., Shen, W., Min, L.J., Hao, Z.H., 2016. Regulation of egg quality and lipids metabolism by zinc oxide nanoparticles. Poult. Sci. 95 (4), 920–933.