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Proteome analysis of egg yolk after exposure to zinc oxide nanoparticles

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are promising candidates as animal diet additive. However, several studies have reported that ZnO NPs cause adverse effects on organisms. Hen egg yolk proteins play vital roles during embryonic development. Although we found ZnO NPs altered the function of the ovary and liver, the effects of ZnO NPs on egg yolk proteins are not as yet understood. In this report, egg yolk proteome was investigated after ZnO NPs treatment. A total of 37 proteins were specifically regulated just by ZnO-NP-50 mg/kg, and 22 proteins were changed solely by $ZnSO_4$-50 mg/kg. Seventeen proteins were regulated by both ZnO-NP-50 mg/kg and $ZnSO_4$-50 mg/kg treatments. Furthermore, the proteins changed by ZnO NPs or $ZnSO_4$ were enriched into different functional groups, respectively, by GO analysis and KEGG pathway enrichment. For the first time, this investigation reports that intact NPs produce a different impact on the egg yolk proteome compared to that of Zn^{2+}. The changes in protein levels by ZnO NPs in egg yolk might influence the value of egg yolk as nutrient and the embryonic development.

1. Introduction

Chicken egg yolk consists of approximately 48% water, 33% lipid, and 17% protein [1]. Egg yolk has the biological function of providing lipids, protein, vitamins, and minerals for embryonic development. Most of the egg yolk constituents are synthesized in the liver under the control of estrogen, are transported in the blood to the ovary, and incorporated into the egg cell by receptor-mediated endocytosis [1–5]. The majority of egg yolk proteins are phosphor-glycoproteins such as low density lipoprotein (LDL), and vitellogenin (VTG). These proteins are in rich of amino acids, phosphate, and carbohydrate, and they also transport lipids, vitamins, and mineral ions into the egg cells [2]. Furthermore, the accumulated maternal antibodies in the egg yolk provide the first line of immune defense for the embryo [6]. In addition to its important roles in reproductive function, egg yolk is also broadly used in the food and cosmetic industries as a binding agent, emulsifier, and natural antioxidant [7–10]. Therapeutic applications have also been suggested, and yolk immunoglobulins have been found widespread used in life sciences [10]. Some egg proteins have also been applied as important raw materials for the production of bioactive peptides [9,10]. Therefore, egg yolk proteins are very important for both the chickens themselves and for humans. The proteome of egg yolk has been investigated previously [10,11] however the effects of zinc oxide nanoparticles (ZnO NPs) on the egg yolk proteome has not yet been reported.

Zinc (Zn) is an essential trace element for humans and animals and it plays various physiological functions in growth and health. It is also involved in female reproduction and studies have found that it is necessary for normal ovulation and fertilization [12]. As an

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integral component of proteins, Zn functions to maintain the structural integrity of about 3000 transcription factors and is essential for the biological activity of more than 300 zinc metalloenzymes [13,14]. Since zinc functions in so many biological activities but the raw materials of hen feeds have low zinc contents, additional supplementation of zinc Zn is required to formulate the diets for hens. As a result, excess Zn is released to the environment and results in environmental pollution [15]. New forms of Zn as diet additive for hens are needed. ZnO NPs are useful nano-materials and have been used in numerous industrial products (e.g., plastics, ceramics, glass, cement, rubber, paints, and pigments). Most impressively, ZnO NPs have been widely used as metal oxide NPs in medical disinfection to inhibit the growth of bacteria [16], fungi [17], and viruses [18,19]. Furthermore, they have been used in personal care products such as sunscreens and cosmetics because they have excellent UV absorption and reflective properties [20]. Due to their small size, ZnO NPs are readily absorbed and are able to cross biological barriers [21], which make them promising candidates as food additive. It has also been reported that ZnO NPs enhance animal performance in chickens and pigs [15]; however, several studies have reported that they cause adverse effects on mice [22], rats [23], fish [24], and Daphnia magna [25]. In our recent studies, we found that ZnO NPs decrease egg yolk lipids [26] and regulate specific gene expression in hen ovary granulosa cells compared to $ZnSO_4$ in vitro [27] and in vivo [28].

Therefore, the hypothesis of this study was that intact ZnO NPs may affect the protein levels in egg yolk after treatment in vivo. The objective of the present investigation was to explore the effects of intact NPs on the proteome in egg yolk and the functional classifications of the altered proteins. $ZnSO_4$, a common diet additive for farm animals, was used as a control as it provides sole Zn^{2+} in the diet.

2. Materials and methods

2.1. ZnO nanoparticle characterization

ZnO NPs were synthesized by Beijing DK Nano Technology Co. LTD (Beijing, P. R. China). The characterization of ZnO NPs has been reported in our recent paper [26].

2.2. Study design (diets and treatments)

All animal experimental procedures followed the regulations of the animal ethics committee of Qingdao Agricultural University [26]. The experiment was 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 on average with free access to food and water. The composition of the basal diet (corn-soybean base) has been previously reported [26,29]. The feeding time was divided into pre-mature (6–18 wk of age) and mature (19–30 wk of age) stages. At these two stages, the diets had a little difference due to nutritional requirements [Supplemental Table 1 (Table S1)]. The hens started to lay eggs at 18 wk of age. To compare the different effects of intact NPs and Zn^{2+}, $ZnSO_4$ was used in this investigation to provide sole Zn^{2+}. In our recent studies, it was found that the Zn content of liver, yolk [26], and blood (unpublished data) samples were similar for $ZnSO_4$-50 mg/kg and ZnO-NP-50 mg/kg treatments after 24 wk of feeding. Furthermore, the regular concentration of Zn used in animal diets is ~40 mg/kg [15]. Therefore, $ZnSO_4$-50 mg/kg and ZnO-NP-50 mg/kg treatments were used in this study [based on the concentration of Zn (mg/kg diet)], and a control treatment (no Zn added, just basal diet) was also included. The Zn content of the basal diet was quantified to be 11.98 ± 0.23 mg/kg. Pullets (n = 144) were randomly assigned to 3 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 30 wk of age, 24 eggs from each treatment (at 10:00 a.m. of one day) were collected to quantify the protein and then 12 hens from each treatment were humanely killed and organ samples were collected for determination of Zn content and intact NPs.

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

The sample preparation for TEM and EDS analyses was followed the previously published procedures [26]. Briefly, organ samples were collected and fixed for 2 h in 2% glutaraldehyde diluted in sodium phosphate buffer (pH 7.2). The specimens were washed extensively to remove excess fixative and subsequently post-fixed in 1% OsO_4 for 1 h in the dark. After extensive washing in phosphate buffer, the samples were dehydrated in an increasingly graded series of ethanol and infiltrated with an increased 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 cut on a Leica Ultracut E equipped with a diamond knife (Diatome, Hatfield, PA), and collected on form var-coated, carbon-stabilized molybdenum (Mo) grids. The section-containing grids were stained with uranyl acetate, allowed to air dry overnight, and imaged on a JEM-2010F TEM (JEOL Ltd., Japan). ZnO nanoparticles in the cells were confirmed using X-Max^N 80 TLE EDS (Oxford Instruments, UK).

2.4. Measurement of Zn concentration in tissues

The methods for quantification of Zn in liver, ovary, yolk, and blood samples have been published in our recent paper [26]. Briefly, tissue samples were weighed and digested in acid and Zn content was determined by Inductively Coupled Plasma atomic Emission Spectroscopy (ICP-OES).

2.5. Effects of ZnO on proteins detected by quantitative proteomics (isobaric tag for relative and absolute quantitation, iTRAQ)

The methodology used to quantify the levels of global proteins in egg yolk has described in our previous report [27], and other publications [30,31].

2.6. Protein extraction and protein digestion

The yolks of 24 eggs from each treatment were homogenized and 1 g of yolk from the mix was lysed in lysate buffer containing phenylmethanesulfonyl fluoride (PMSF, 1 mM), ethylenediaminetetraacetic acid (EDTA, 2 mM), and DL-Dithiothreitol (DTT, 10 mM) on ice for 5 min and sonicated on ice for 5 min. Then the samples were centrifuged at 30 000 ×g for 20 min at 4 °C, and the supernatant was collected. Five times of cold acetone was added to the supernatant with a final amount of 10 mM DTT and the mix was stored at 4 °C overnight to precipitate the proteins. Samples were then centrifuged at 30 000 ×g for 20 min at 4 °C, and the supernatant was discarded. The protein pellets were washed with cold acetone with the final 10 mM DTT remaining at −20 °C for 30 min; it was then centrifuged at 30 000 ×g for 20 min at 4 °C. The pellets were washed twice and briefly dried to remove the acetone. The protein pellets were again lysed in lysate buffer containing PMSF (1 mM), EDTA (2 mM), and DTT (10 mM) on ice for 5 min and