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Effects of Zinc Oxide Nanoparticles on Neural Tube Development in Early Chicken Embryos

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ABSTRACT

AIM: To investigate the effect of ZnO-NPs on neural tube development in early chicken embryos.

MATERIAL and METHODS: Fifty pathogen-free fertilized eggs were initially incubated for thirty hours. The eggs were divided into 5 groups. In the control group (C) the egg's apex was opened and closed without any administration. In the distilled water group (DW), 10 microliters of distilled water were injected into the sub-blastodermic area. ZnO-NP suspensions were prepared in distilled water and injected sub-blastodermically into the low, medium and high dose ZnO-NP groups (10 mg/kg, 30 mg/kg, and 50 mg/kg, respectively). Incubation was completed in 72 hours, and embryological and neural tube development was evaluated histologically with a light microscope.

RESULTS: Embryos in all groups were evaluated according to the Hamburger-Hamilton (HH) staging. It was observed that the staging progressed by the developmental process between 68-72 hours, which is equivalent to the 19-20th stage of HH. Differentiated otic vesicle, optic cup, lens vesicle, pharynx, and Rathke's pouch were all observed in embryo sections. Both forebrain and hindbrain vesicles were easily distinguished in the sections by cranial flexion. Neural tube closure defect was not detected in any of the groups.

CONCLUSION: In our observations, ZnO-NPs did not affect neural tube development at the applied dose ranges. We believe that additional studies with higher doses using a higher number of subjects will help clarify the conflicting data in the literature.

KEYWORDS: Chicken embryo, Neural tube development, Zinc oxide, Zinc oxide nanoparticle

ABBREVIATIONS: ZnO: Zinc oxide, **FDA:** Food and Drug Administration, **ZnO-NP:** zinc oxide nanoparticles, **HH:** Hamburger-Hamilton, **NTD:** Neural tube defects

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INTRODUCTION

Zinc oxide (ZnO) is an insoluble mineral in a white powder form. It is a mineral filter approved by the American Food and Drug Administration (FDA). Recently, zinc oxide nanoparticles (ZnO-NP) smaller than 100 nanometers in diameter have been in common use. ZnO-NPs, is one of the most widely used nanoparticles used in toothpastes, cosmetics, sunscreens, diaper rash creams, coatings, sports equipment, tires, and electronics. In addition, potential application fields of ZnO-NPs are increasing, including agricultural water purification and dietary supplements (1,2,7,15,25,26). However, the risk and mechanism of toxicity in humans are not well known. The exposure of pregnant women to ZnO-NPs is increasing daily due to its widespread usage. Studies also show that some nanoparticles may pass through the placenta (13,14). Therefore, when a mother is exposed to ZnO-NPs during pregnancy, the embryo may also be exposed to ZnO-NPs. However, it is unclear whether this situation has a side effect on embryonic development.

Neural tube defects (NTDs) represent an important group of congenital anomalies. Every year, about 300,000 children are born with NTDs worldwide; nearly 88,000 die, while the remainder survive with some disabilities. The global frequency of NTD is between 1-10 per 1000 births (11). In Turkey, this rate was reported to be 3-5,8/1000 (23). It has been known for a long time that environmental factors such as folic acid deficiency, maternal diabetes or high glycemic index also have a role in this anomaly. Exposure to teratogens, especially during the neurulation phase of embryogenesis, may result in fetal neural tube closure defects (21). Since teratogenic, toxic effects and dose determination studies cannot be performed in humans, toxic substances are tested in the laboratory animals. Since the early development of the chicken embryo resembles the first month of human embryonic development, there are many studies on chicken embryos (3,5,8-10,16-20,24-26). Therefore, our study is aimed to determine whether ZnO-NPs deteriorate neural tube development in early chicken embryos.

MATERIAL and METHODS

This study was approved by Başkent University Institutional Review Board (Project no: DA22/07) and supported by Başkent University Research Fund. Fifty pathogen-free, fertilized chicken eggs (ATAK-S strain, mean ± SD, 50 ± 4 g) were obtained from the Ministry of Agriculture and Forestry, Directorate of Poultry Research Institute (Yenimahalle/Ankara). The eggs were placed in an automatic cycle incubator with the appropriate temperature and humidity conditions (37.8 °C ± 0.2 °C and 55–60%, respectively) (Figure 1A) (3,16). The eggs were divided into five groups (n =10): control group (C), distilled water group (DW), low dose (LD), medium dose (MD), and high dose (HD) zinc oxide nanoparticle groups (ZnO-NP: Sigma-Aldrich, 544906, nanopowder < 100nm particle size). Drug administration to the embryos was performed at the 30th hour of incubation (HH 9) stage, when neural folds began to form according to Hamburger-Hamilton (HH) staging (6). At the 30th hour of incubation, the apices of the eggs were opened by sterile surgical instruments (Video 1). The apex of the eggs in the control group was opened and closed with sterile surgical tape without any operation. In the distilled water group (DW), 10 microliters of distilled water were injected into the sub-blastodermic area with a Hamilton syringe (26s ga 2in2) and the apex of the egg was closed with sterile surgical tape (Video 2). Sub-blastodermic injections of 10 microliters ZnO-NP suspensions were applied to the low, medium and high dose ZnO-NP groups (10 mg/kg, 30 mg/kg and 50 mg/kg, respectively). To ensure adequate dispersion of the NPs, the suspension was vortexed and sonicated for 30 minutes using an ultrasonic cleaner (Sonorex RK 52H) prior to the injections (26). After the injections were completed, all groups were closed with sterile surgical tapes and placed back in the automatic cycle incubator. The incubation was terminated at 72 hours when both the anterior and posterior neuropores were expected to be closed. At this stage, the viability of the embryos was evaluated by the presence of the heartbeat (Video 3). Then, euthanasia was performed by injecting a high-dose anesthetic containing pentobarbital, and the embryos were collected (Figure 1B, C). The embryos were fixed in a 2% glutaraldehyde solution, and then plastic blocks were prepared. Semi-thin sections were taken from the plastic blocks and dyed with toluidine blue. Sections were examined and photographed under light microscope (Leica DM 3000) connected to a digital camera (Leica DFC 500).

Statistical Analysis

For the evaluation of the data, mean and standard deviation values were used as descriptive statistics for qualitative assessment, and frequency (N) and percentages (%) were used for quantitative evaluations. For comparative analyses, the Fisher's exact test was used (Table I). The statistical significance was considered to be $p<0.05$. All the analyses were conducted with an R V4.2.2 environment for statistical computing (22).

RESULTS

Each group of eggs was checked by opening a window at their apex at the 30th hour of unfertilized eggs. In this control, it was observed that all eggs were fertilized. In the control group, the eggs were closed without any treatment as a sham group. At the 72nd hours of the experiment, in the control group 8 live embryos out of 10 were detected. Furthermore, in the distilled water group, 9 live embryos out of 10 were detected. In zinc oxide NP applied groups; 9 live embryos were detected in the low-dose group, 8 live embryos in the medium-dose group, and 9 live embryos in the high dose group. For all the groups, the remaining embryos did not develop (Table I). Microscopic evaluations were made on sections taken from different planes of interest. All the embryos were evaluated according to the Hamburger Hamilton staging system and it was determined that their development was compatible with the HH 19-20 stage, which corresponds to the 68-72nd hours of staging (6). Consistent with this stage, neural tube development, development of pharyngeal arches and heart, formation of otic and lens vesicles, and optic cups were observed in all embryos (Figure 2, 3). While the suitability of

Table I: Zinc Oxide Distribution among the Groups

Groups	Control n (%)	DW n (%)	LD n (%)	MD n (%)	HD n (%)	p-value *(<0.05)
Unfertilized	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Live embryo	8 (80)	9 (90)	9 (90)	8 (80)	9 (90)	
Undeveloped	2 (20)	1 (10)	1 (10)	2 (20)	1 (10)	1.00
Neural tube defect	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Total	10 (100)	10	10	10	10	

Fisher's Exact test. ***DW:*** *Distilled water,* ***LD:*** *Low-dose,* ***MD:*** *Middle-dose,* ***HD:*** *High-dose ZnONPs.*

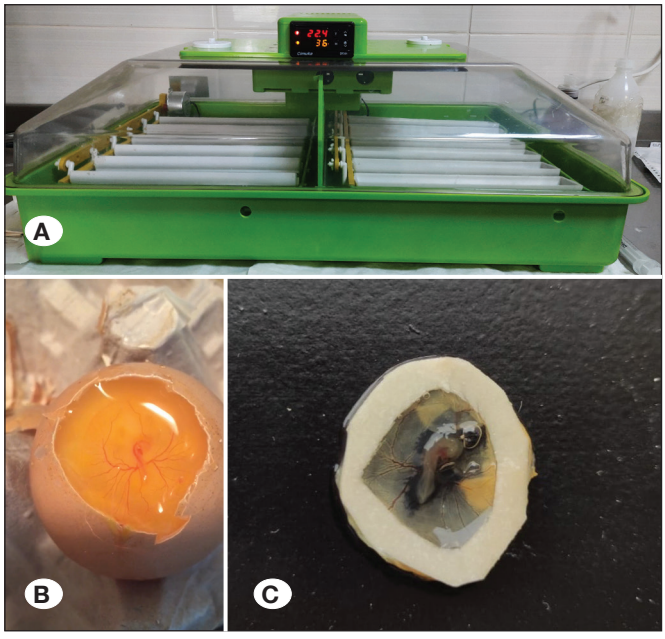

Figure 1: A) Automatic cycle incubator **B)** Image of the embryo in the egg with the apex opened at 72 hours **C)** embryo collection process.

embryological development was evaluated with the embryos in the control group, the propriety of the experimental method was evaluated with the distilled water group. Due to the head-neck flexure of the embryos, both forebrain and hindbrain vesicles were observed in the sections (Figure 2). The neural tubes of all embryos in each group were examined, and open neural tube was not detected in any of the groups (Table I) ($p>0.05$).

DISCUSSION

Neurulation, which is an essential and complex process involving molecular mechanisms, takes place during the period when the embryo is very sensitive to teratogens. If a pregnant woman is exposed to a teratogen during this period, neural tube defects may occur. With the widespread use of nanoparticles, ZnO-NPs have become a popular compound that pregnant women may frequently encounter in cosmetics, sunscreens and food products. Side effects of some nanoparticles on reproductive organs were previously reported (4,12).

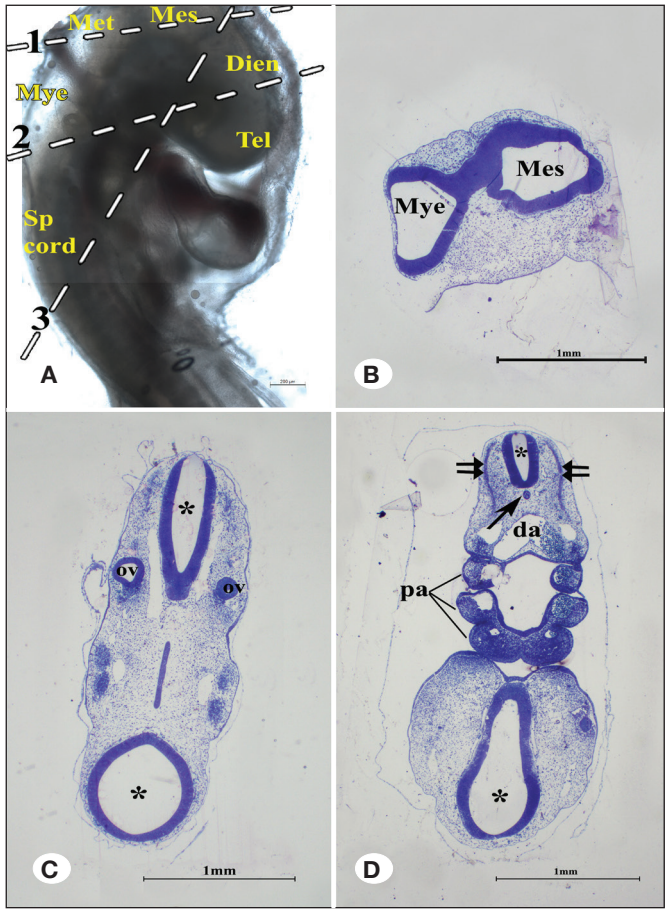

Figure 2: A) Macroscopic view of the embryo. This figure was created by merging 3 separate micrographs in Adobe Photoshop program. Micrographs were taken from the collected embryos at X5 magnification without any processing. Spinal cord (sp cord) and primary brain vesicles were described as Tel; Telencephalon, Dien; Diencephelon, Mes: Mesencephalon, Met; Metencephalon, Mye; Myelencephalon. Dashed lines indicate the levels of the sections seen on figures 2b, 2c and 2d. **B)** The section through the dashed line number 1. Both closed mesencephalon (Mes) and myelencephalon (Mye) and also connection of these two brain vesicles were seen in the tangential section of the embryo (Toluidin blue, X4). **C)** The section through the dashed line number 2. Two sections of the closed neural tube (*) were seen because of the head-neck flexure of the embryos. Otic vesicles (ov) were distinguished unequal in size due to sectional asymmetry (Toluidin blue, X4). **D)** The section through the dashed line number 3. Two sections of the closed neural tube (*), bilateral somites (double arrow), notochord (arrow), ductus arteriosus (da) and pharyngeal arches (pa) were observed (Toluidin blue, X4).

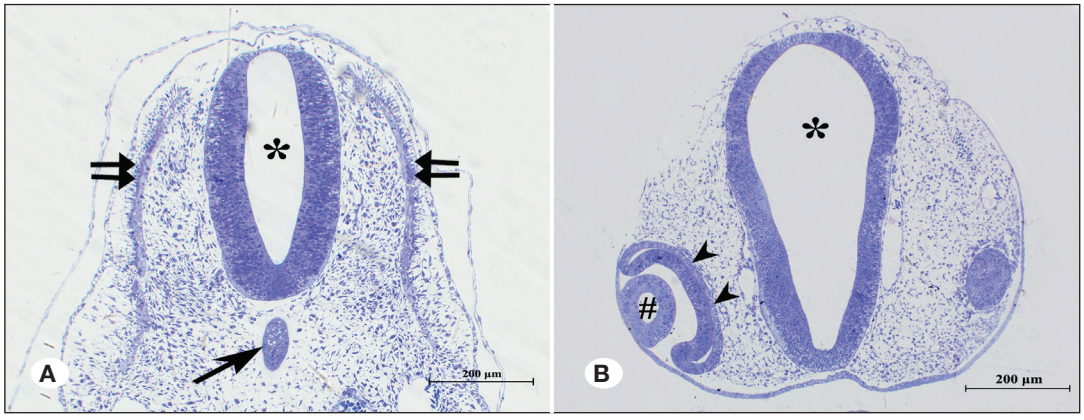

Figure 3: A) Higher magnification of the already closed neural tube section (*) through diencephalon. Notochord (arrow) and somites on both sides (double arrows) were also observed in detail (Toluidin blue, x10). **B)** Another section through closed neural tube (*) also demonstrating optic cup (arrowhead) and lens vesicle (#) clearly on one side. On the opposite side tangential section of optic cup was seen because of the sectional asymmetry (Toluidin blue, x10).

Due to their small size and ability to enter cells by endocytosis, it is suggested that the potential teratogenic risk of ZnO-NPs may increase. Since the early development of the chicken embryo is similar to the first month of human embryonic development, early chicken embryos were used in this study (3,9,10,16-19,24-26). Seasonal changes, incubation conditions and genetic factors can cause variations in chicken embryo development (6). For this reason, a control group without any intervention was also included to follow the developmental process. A distilled water group was established to determine the possible effect of injection on embryo development. Ten microliters of distilled water were applied sub-blastodermically to equalize the total volume made in the injection. Doses of zinc oxide NP solutions have been studied as low, medium, and high doses per the literature (1,2,7,14,15,25,26). The developmental process was found to be consistent with the Hamburger Hamilton staging. It was shown that physical conditions and experimental methods did not impair embryonic development. All embryos in all groups were examined, and no neural tube defect was detected. In the literature, studies are conducted on different animals with the application of ZnO-NPs. Abbasi et al. gave dietary ZnO-NPs to Japanese quail and found gonadal dysfunction (1). Another group worked with the Javanese medaka embryos in the ultra-pure, deionized and dechlorinated tap water to explore lethal doses of the ZnO-NPs. They pointed out that the mortality rate of Javanese medaka embryos increased as the concentration of ZnO-NPs increased in all types of water. There was a strong correlation between the time of exposure and the mortality of embryos (2). In the study where pregnant rats were given dietary ZnO-NP, it was reported that maternal weight decreased, but no teratogenicity was found in fetuses (7). There are different results in the literature in studies with chick embryos. A group of researchers injected hydrocolloids of ZnO-NPs on the first day of incubation in concentrations of 50, 100, and 500 mg/L into the air sac of the fertilized eggs and compared the body and organ weights of the animals after a 19-day incubation period. They reported that injection of ZnO-NPs into air sac of the eggs at the beginning of hatching did not affect the body weight, survival rate and health status of embryos in the final stage of embryogenesis. In the second phase of the study, they replaced ZnO-NPs with ZnO in the diets of chicks and showed that ZnO-NPs did not harm chicken growth (15). Another group injected 100 µl 50µg/ml ZnO-NP into the air chamber of the chicken embryos every day from the day zero until day nine or twelve. The surviving embryos were harvested to analyze the cardiovascular, nervous, and skeletal systems. Their results showed that ZnO-NPs caused the different degrees of deformity in the craniofacial skeleton, including the shortened or curved coracoids and the shrinkage or absence of parietal bone (25). The same group explored whether ZnO-NPs exposure caused proportional growth failure of neural tube closure in mouse and chicken embryos. They also demonstrated this growth failure in several cell lines. HH0 chicken embryos were incubated in a humidified incubator at 38 °C in a humidified atmosphere in the absence or in the presence of 12.5 µg/ml, 25 µg/ml, and 50 µg/ml ZnO-NPs until the HH10 stage in their study. Surviving embryos were harvested at the HH10 stage. They suggested that exposure to ZnO-NPs during pregnancy is a considerable risk factor for congenital diseases, such as failure of neural tube closure (26). In this study, we injected the ZnO-NPs into the sub-blastodermic area of the chicken embryos instead of the air sac to exclude diffusion problems. We terminated the incubation at HH19-20 stage to ensure that neurogenesis was complete. We found that ZnO-NPs did not cause neural tube closure defects and did not impair embryonic development under these circumstances.

CONCLUSION

In our study, embryo development in all groups was found to be compatible with age, and neural tube defect was not observed. However, considering the available literature data, it is thought that further studies with higher doses need to be performed before claiming that nanoparticle usage is clearly safe.

AUTHORSHIP CONTRIBUTION

Study conception and design: PAF
Data collection: EE, YH, BNC, SC, RSO
Analysis and interpretation of results: EE, UT
Draft manuscript preparation: EE
Critical revision of the article: YH, BNC, SC, RSO
Other (study supervision, fundings, materials, etc...): JA
All authors (PAF, EF, UT, JA, YH, BNC, SC, RSO) reviewed the results and approved the final version of the manuscript.

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