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Prenatal development toxicity study of zinc oxide nanoparticles in rats

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Abstract: This study investigated the potential adverse effects of zinc oxide nanoparticles ([$ZnO^{SM20(+)}$ NPs] zinc oxide nanoparticles, positively charged, 20 nm) on pregnant dams and embryo–fetal development after maternal exposure over the period of gestational days 5–19 with Sprague-Dawley rats. $ZnO^{SM20(+)}$ NPs were administered to pregnant rats by gavage at 0, 100, 200, and 400 mg/kg/day. All dams were subjected to a cesarean section on gestational day 20, and all of the fetuses were examined for external, visceral, and skeletal alterations. Toxicity in the dams manifested as significantly decreased body weight after administration of 400 mg/kg/day NPs; reduced food consumption after administration of 200 and 400 mg/kg/day NPs; and decreased liver weight and increased adrenal glands weight after administration of 400 mg/kg/day NPs. However, no treatment-related difference in: number of corpora lutea; number of implantation sites; implantation rate (%); resorption; dead fetuses; litter size; fetal deaths and placental weights; and sex ratio were observed between the groups. On the other hand, significant decreases between treatment groups and controls were seen for fetal weights after administration of 400 mg/kg/day NPs. Morphological examinations of the fetuses demonstrated significant differences in incidences of abnormalities in the group administered 400mg/kg/day. Meanwhile, no significant difference was found in the Zn content of fetal tissue between the control and high-dose groups. These results showed that oral doses for the study with 15-days repeated of $ZnO^{SM20(+)}$ NPs were maternotoxic in the 200 mg/kg/day group, and embryotoxic in the 400 mg/kg/day group.

Keywords: developmental toxicity, maternal toxicity, nanotoxicology, teratogenicity

Introduction

Recent advances in nanotechnology have spurred increases in the use of nanoparticles (NPs), and concerns over the possible detrimental effects associated with exposure to NPs.[1] Zinc oxide nanoparticles (ZnO NPs) are currently engineered and most widely used NPs. Most applications with ZnO powder exploit the reactivity of the oxide, as a precursor to other zinc compounds.[2] The applications in material science utilize the high refractive index, high thermal conductivity, and binding properties of ZnO. Possible exposure to ZnO NPs could occur in the industrial settings and through everyday consumer products. ZnO NPs are added into diverse materials and products, such as plastics, ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesive, sealants, pigments, batteries, ferrites, and fire retardants. In addition, ZnO nanomaterials possess ultraviolet (UV)-shielding, antibacterial properties, deodorizing effects, and heat and UV light resistance, which could provide many great potentials for a wide range of applications in many fields: cosmetics and sunscreens,[3] food additives, additives in packing,[4,5] fungicides in agriculture,[6] and biomedical applications such as anticancer drugs.[7,8] However, the human risk and toxicity mechanism are not well known.

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Since zinc is an essential trace element in the human body and is commonly present in foods or added as a nutritional supplement, ZnO is generally considered to be a material with low toxicity.[9] However, ZnO could turn into hazardous material upon inhalation as a gas (for example, metal fume fever), since fumes could be generated from melting and oxidizing at high temperature from zinc or zinc alloys.[9] Drinker et al[10] and Balance et al[11] reported that higher concentrations of freshly generated ZnO, as given in previous human inhalation exposure studies, can produce symptomatic, physiologic, and hematologic effects, as well as elevations in certain peripheral blood and bronchoalveolar lavage cytokines. It should be noted that small-sized particles are more reactive and responsive than bulk-sized particles, because they have a higher proportion of atoms on their surface.[12] Also, due to the lower surface energy of ZnO NPs, they can be well dispersed in various solvents as well as in air.[13] Therefore, exposures and uptakes of ZnO NPs could occur through various routes.

Few reports were published on the diverse biological systems of ZnO NPs.[13,14] Severe damages to liver and lung tissues from acute inhalation of ZnO NPs at dose of 2.5 mg/kg body weight in Wistar rats were reported by Wang et al.[14] Also, nano-forms of various particles were more toxic than their micro-counterparts after acute exposure via the oral route in mice.[15] In an another study by Wang et al, acute oral toxicity study in mice of ZnO NPs at a high dose range (1–5 g/kg body weight) revealed the increased damages to liver, spleen, and pancreas with increased doses.[16] In the dermal toxicity study, 28-day repeated dose of ZnO NPs caused the greater collagen losses in skin of Sprague-Dawley (SD) rats in comparison with tail.[17] Liver, lung, and kidney were considered to be the main target organs in a pharmacokinetic and tissue distribution study.[18] Also, Lu et al[19] suggested that ZnO NPs could disturb the energy metabolisms and cause impairments in mitochondria and cell membrane of rat kidney, in causing ZnO NPs-induced nephrotoxicity. In spite of significant impact of increased usages and productions of ZnO NPs to human health and the environment, the potential adverse effects of ultrafine ZnO on pregnant dams and embryo–fetal development have never been determined.

Currently, there is a serious lack of information on the potential NP hazard to human health, particularly on their possible toxic effects on the endocrine system, and existing data and knowledge of potential endocrine interactions and toxicities are quite limited (for example, reprotoxicity).[20,21] Some studies of reproductive function suggest that exposure to some nanomaterials may disrupt endocrine functions such as regulation of serum sex-hormone levels. In contrast, other nanomaterials may prevent endocrine dysfunction via various mechanisms, including antioxidant effects.[20] Also, a lot of evidence shows that fetuses are affected more than adults by a variety of environmental toxins because of physiological immaturity. Yamashita et al[21] reported that nanosilica induced fetal resorption and restricted fetal growth, and surface modification of nanosilica with carboxyl or amine groups prevented resorption and fetal growth restriction in the study of nanosilica (70 nm).

Therefore, studies of reproductive function are necessary to evaluate the potential endocrine-disrupting risks and the effects on fetuses and pregnancies of nanomaterials. The present study was undertaken to investigate the potential adverse effects of 20-nm positively charged ZnO NPs ($ZnO^{SM20(+)}$ NPs) on pregnant dams and embryo–fetal development in SD rats. The results of this investigation could provide additional relevant information to the safety evaluation of $ZnO^{SM20(+)}$ NP exposures during pregnancy in SD rats.

Materials and methods

This study was performed in compliance with Organization for Economic Cooperation and Development (OECD) test guideline 414 entitled to Prenatal Developmental Toxicity Study,[22] and in accordance with the Good Laboratory Practice (GLP) principle.[23,24] The GLP process was performed in accordance with the standard operation procedures certified by the Ministry of Food and Drug Safety (MFDS). All of the animals were cared for as specified in the "Guide for the Care and Use of Laboratory Animals" issued by the Animal Care and Use Committee of the NVRQS (National Veterinary Research and Quarantine Service).

Characterization of ZnO NPs

ZnO^{SM20} NPs (Lot No 141319 for 20 nm), supplied by Sumitomo-Osaka Cement Co. (Tokyo, Japan), had 100.1% assay analysis and also contained <1 ppm Fe not detected and 1.5 ppm arsenic trioxide (As2O3). The ZnO NPs were capped with L-serine molecules, which are widely used capping agents for in organic NPs, providing charge surface property.[25–27]

Animals and dosage

Crl:CD(SD) rats are commonly used in toxicity studies, as they have a large amount of reference data accumulated over a long time. Male and nulliparous female rats at 10 weeks of age were obtained from a specific pathogen-free colony at Orient Bio Inc. (Gyeonggi-do, South Korea) and used after 12 days of quarantine and acclimatization. The animals were

housed in a room that was maintained at a temperature of 20.8°C–23.0°C and a relative humidity of 45.3%–56.9%, with artificial lighting from 8 am to 8 pm and 10–15 air changes per hour. Normally, 1:1 (one male to one female) mating was used in this study. Each morning, the female rats were examined for the presence of sperm or a vaginal plug. Day 0 of pregnancy was defined as the day when a vaginal plug or sperm was found. The mated females were housed individually in clear polycarbonate cages with stainless steel wire lids. They were allowed to drink sterilized tap water with UV irradiation and fed on commercial rodent chow (Cargill Agri Purina, Gyeonggi-do, Korea) ad libitum.

For the oral administration of $ZnO^{SM20(+)}$ NPs, the NPs were suspended in 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer with 2% L-serine (vehicle) and then mixed well. The final pH of the buffer solution was adjusted with hydrochloric acid to 6.5, and 20% of the surface-modified $ZnO^{SM20(+)}$ NPs were used as a stock solution. Before the administration, the suspension was stirred for 10 seconds and then diluted with distilled water. Concentration of dosing solution was measured on gestational day (GD) 5, 11, and 19 using inductive coupled plasma atomic emission spectrometry (ICP-AES) (ULTIMA2; HORIBA JOBIN YVON, Paris, France).

The ZnO NPs were administered daily by gavage to pregnant rats from GD 5 through 19 with a dose volume of 10 mL/kg body weight. The vehicle control group received only HEPES/L-serine buffer solution with gavage. The daily application volume was calculated in advance based on the most-recently recorded body weight of the individual animal.

Dose-range finding study

In a previous dose-range finding study, 14-day repeated oral dose of $ZnO^{SM20(+)}$ NPs showed decreases in body weight, and changes in hematology and biochemistry parameters for 1,000- and 2,000-mg/kg/day groups. Death occurred in two male rats from the 2,000-mg/kg/day group. Decreases in body weight, reductions in food consumption, and changes in hematology and biochemistry parameters were observed from 1,000- and 2,000-mg/kg/day groups. In a subchronic toxicity study of 90-day repeated oral treatment of $ZnO^{SM20(+)}$ NPs, the decreases in food and water consumptions were observed in 125-, 250-, and 500-mg/kg/day groups. Reductions in thymus weight, atrophy of testis and seminal vesicle, and changes of hematology and biochemistry parameters were present in 250- and 500-mg/kg/day groups. Moreover, repeated oral doses of $ZnO^{SM20(+)}$ NPs caused aninar cell apoptosis, submucosal edema, inflammation in the glandular stomach, and decreases in total protein and albumin for 250- and 500-mg/kg/day groups. Either single oral dose or 90-day repeated oral doses (vehicle control and 125, 250, and 500 mg/kg/day) was performed for pharmacokinetic study; the increased zinc concentrations in plasma were seen for 125-, 250-, and 500-mg/kg/day groups.[28] Therefore, for the prenatal and developmental toxicity study, the high dose was set to 400 mg/kg/day of body weight, and the middle and low doses was set to 200 and 100 mg/kg/day, respectively.

Experimental groups

A total of 91 healthy female rats were assigned randomly to four experimental groups as follows: three treatment groups of $ZnO^{SM20(+)}$ NPs receiving 100 (n=24), 200 (n=21), and 400 mg/kg/day (n=23), and a vehicle control group (inseminated females per group). The selected doses for this study were based on the results of a dose-range finding study, conducted in our laboratory.

Observation of dams

All pregnant females were observed daily throughout the gestation period for clinical signs (mortality, morbidity, general appearance, and behavior). Maternal body weights were measured daily from GD 0–20, and individual food consumptions were determined on GD 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18. At the scheduled termination day (GD 20), all of the pregnant females were euthanized by isoflurane inhalation and exsanguination from the aorta. A complete gross postmortem examination was then performed. The absolute and relative (organ-to-body weight ratio) weights of the liver, heart, brain, kidneys, ovaries, spleen, lung, uteral cornua, adrenal glands, and pituitary were measured.

Postmortem examination

The ovaries and uteri of each female were removed and examined for the number of corpora lutea and the status of all the implantation sites, ie, live and dead fetuses, early and late resorptions, and total implantations. Uteri with no evidence of implantation were stained with a 2% sodium hydroxide solution to identify the presence of early resorption sites.[29] If no stained implantation site was present, the rat was considered "not pregnant".

On day 20 of gestation, dams were subjected to cesarean section. Following measurement of gravid uterine weight, corpora lutea, implantation, live fetuses, fetal resorptions,

and dead fetuses were counted and recorded. Based on the results, the following were calculated:

$$\text{Preimplantation loss (\%)} = \frac{\text{(number of corpora lutea} - \text{number of implantations)}}{\text{(number of corpora lutea)}} \times 100 \quad (1)$$

$$\text{Postimplantation loss (\%)} = \frac{\text{(number of implantations} - \text{number of live fetuses)}}{\text{(number of implantations)}} \times 100 \quad (2)$$

$$\text{Fetal death} = \text{resorptions} + \text{dead fetuses} \quad (3)$$

Resorption was classified as "early" when only a resorption site resembling a dark brown blood clot and with no embryonic tissue was visible, and was considered "late" when both the placental and embryonic tissues were visible at the postmortem examination. All live fetuses were weighed individually, sexed, and examined for any morphological abnormalities, including a cleft palate. Alternate fetuses were selected for either skeletal or visceral examinations. Half of the live fetuses from each litter were fixed in absolute ethanol, eviscerated, and then processed for skeletal staining with alizarin red S and Alcyan blue using for subsequent skeletal examination.[30] The other half was preserved in Bouin solution and examined for internal soft-tissue changes using a freehand razor sectioning technique[31] and Nishimura's method.[32] The observed fetal morphological alterations in this study were classified as developmental malformations or variations. A malformation was defined as a permanent structural change that is likely to adversely affect survival or health.[33] The term "variation" was defined as a change that occurred within the normal population under investigation and may be unlikely to adversely affect survival or health. Terminology suggested in an internationally developed glossary of terms was used to classify the structural developmental abnormalities in common laboratory mammals.[34]

Zn concentration in fetal tissue

To investigate the placenta transfer of $ZnO^{SM20(+)}$ NPs in vivo, four extra female rats were used in the control (control group; n=2) and 400-mg/kg/day groups ($ZnO^{SM20(+)}$ NPs; n=2). Dosing occurred for the period of GD 5–19 in the same manner as that for the main study animals. On GD 20, fetuses were collected via cesarean sections from dams, and Zn contents in the fetal tissues were analyzed. Fetuses were digested in concentrated nitric acid overnight. The next day, nitric acid and perchloric acid were added to each sample and heated at 200°C–250°5 until the solutions were colorless and clear. The concentrated sample solutions were transferred into a 100-mL volumetric flask and filled with purified water to the measuring line. Before analysis, ICP-AES (HORIBA JOBIN YVON) was calibrated every time by running at least six Zn standard concentrations (0.5, 2, 5, 10, 20, and 40 mg/L).

Data analysis

The unit for statistical measurement was the pregnant female or the litter.[35] Quantitative continuous data, such as the maternal body weight, food consumption, fetal body weight, and placental weight, were subjected to a one-way analysis of variance, and a Scheffe's multiple comparison test was carried out for the significant differences.[36] The number of corpora lutea, total implantations, live and dead fetuses, and fetal alterations were evaluated statistically by using the Kruskal–Wallis nonparametric analysis of variance,[37] followed by the Mann–Whitney *U*-test where appropriate. The sex ratio and the proportions of litters with malformations and developmental variations were compared using a chi-square test and Fisher's exact probability test.[38] Statistical analyses were performed by comparing the treatment groups with the control group using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). Differences with a *P*-value of 0.05 or lower were considered to be statistically significant.

Results

Formulations analysis of test article

Table 1 summarizes the concentrations of dosing solution for $ZnO^{SM20(+)}$ NPs on GD 5, 11, and 19. Three analyses confirmed that the analyzed concentrations of all dose formulations were within ±15% of the target concentrations. $ZnO^{SM20(+)}$ NPs were stable for 4 hours at room temperature. Concentrations of total zinc were 9.82±0.84 mg/mL (mean ± standard deviation) for the 100-mg/kg/day group, 20.04±1.63 mg/mL for the 200-mg/kg/day group, and 40.80±1.47 mg/mL for the 400-mg/kg/day group.

Zn concentration in fetuses

Zn concentrations in fetal tissues are shown in Figure 1. The measured total Zn levels by ICP-AES were 14.44±0.37 μg/g (mean ± standard deviation) for the control group and 16.47±2.19 μg/g Zn for the 400-mg/kg/day group (Table 2). The Zn contents in fetuses after in utero exposure to $ZnO^{SM20(+)}$ NPs was not significantly different from the Zn contents in control fetuses.

Table 1 Formulations analysis of dosing solution for $ZnO^{SM20(+)}$ NPs on gestation day 5, 11, and 19

Dosing date	$ZnO^{SM20(+)}$ NPs		
	Target concentration (mg/mL)	Determined concentration (mg/mL)	Difference from target (%)
Gestation day 5	0	Not detected	Not detected
Gestation day 11	0	Not detected	Not detected
Gestation day 19	0	Not detected	Not detected
Gestation day 5	10	10.03	0.31
Gestation day 11	10	8.90	−11.00
Gestation day 19	10	10.53	5.30
		[a]9.82±0.84	
Gestation day 5	20	20.72	3.61
Gestation day 11	20	18.18	−9.10
Gestation day 19	20	21.22	6.10
		[a]20.04±1.63	
Gestation day 5	40	40.53	1.32
Gestation day 11	40	42.39	5.98
Gestation day 19	40	39.48	−1.30
		[a]40.80±1.47	

Note: [a]Values are expressed as mean ± standard deviation.
Abbreviations: NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Effects on dams

Although seven of 24 dams from the 100-mg/kg/day group, 12 of 21 dams from the 200-mg/kg/day group, and 18 of 23 dams from the 400-mg/kg/day group showed salivation around the mouth in general appearance. Starting from 3–12 days after oral administration, alopecia (localized areas of partial alopecia) was observed in one pregnant rat from the 100-mg/kg/day group, one from the 200-mg/kg/day group, and one from the 400-mg/kg/day group (data not shown). This clinical sign was not recovered for the treatment period.

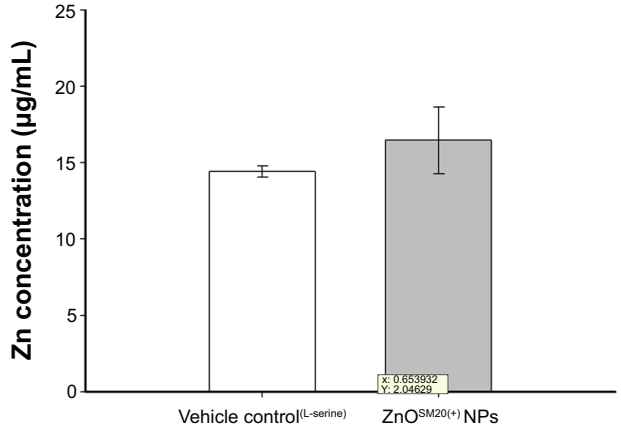

Figure 1 The total Zn levels measured with ICP-AES.
Notes: To investigate the placenta transfer of $ZnO^{SM20(+)}$ NPs in vivo, four extra female rats were used in the control (nontreatment control group; n=2) and 400-mg/kg/day ($ZnO^{SM20(+)}$ NPs; n=2) groups. Dosing occurred on gestational day 5–19 in the same manner as for main study animals.
Abbreviations: ICP-AES, inductive coupled plasma atomic emission spectrometry; $ZnO^{SM20(+)}$ NPs, 20-nm positively charged ZnO nanoparticles.

The changes in body weight during the entire experimental period are listed in Figure 2. As shown by the data in Table 3, significant decreases in maternal body weight on GD 20 from the high-dose group was observed in comparison with the vehicle control group. The maternal-body-weight gain during pregnancy and corrected body weight were also significantly lower in the high-dose group than in the control group. Statistically significant decreases in food consumption were noticed on day 18 of gestation in the 200- and 400-mg/kg/day groups in comparison to the vehicle control group (Table 4). At the scheduled autopsy, one case of caveola of kidney surface in the vehicle control group; one case of splenomegaly in the 200-mg/kg/day group; and hypertrophy of adrenal and lung, edematous bowel, gastro-tympanites, and red reaction of liver in the 400-mg/kg/day group were observed in dams (data not shown). The absolute and relative organ weights of the pregnant rats treated with $ZnO^{SM20(+)}$ NPs are presented in Table 5. Significantly decreased absolute liver weight in the 400-mg/kg/day group was observed, and the increased absolute and relative weights of adrenal gland were significant in the 400-mg/kg/day group in a dose-dependent manner in comparison with the vehicle control group.

Effects on embryo–fetal development

Table 6 summarizes the reproductive findings for the pregnant rats treated with $ZnO^{SM20(+)}$ NPs on GD 5–19. The overall pregnancy rates were similar for all dosage groups, ranging

Table 2 The Zn content in fetuses after in utero exposure to $ZnO^{SM20(+)}$ NPs

Parameters	Unit	Dose (mg/kg bw/day)	
		Nontreatment control group	400 mg/kg treatment ($ZnO^{SM20(+)}$ NPs)
Number of fetuses/pregnant females	(fetus)	28/2	28/2
Analyzed concentration	(μg/mL)	9.04	8.94
	(μg/mL)	6.77	8.82
	(μg/mL)	[a]7.91±1.61	[a]8.88±0.08
Dilution		100.00	100.00
Sample weight	(g)	63.75	49.60
	(g)	46.05	59.10
	(g)	[a]54.90±0.37	[a]54.35±6.72
Conversion concentration	(μg/g)	14.18	18.02
	(μg/g)	14.70	14.92
	(μg/g)	[a]14.44±0.37	[a]16.47±2.19

Note: [a]Values are expressed as mean ± standard deviation.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

from 87.5%–100%. Totally resorbed litters were not found in any group. The number of corpora lutea, implantations, and fetal deaths, as well as implantation rates, placental weight, and sex ratios of the live fetuses were similar for the treatment groups and the vehicle control group. Significantly decreased fetal weights of males and females were observed in the 400-mg/kg/day group in comparison to the vehicle control group. No fetus showed an external malformation. Hematoma was shown in all groups, including the vehicle control group. However, the numbers of fetuses with hematomas were not significantly increased in comparison to the control group (Table 7). Several types of visceral variations were seen in fetuses of the treatment groups, including misshapen thymus, ureter abnormality (grade I: slight dilation of the renal pelvis, grade II: reduced papilla size and noticeable dilation of the renal pelvis, grade III: very short or no papillae and a marked dilation of the renal space),[39] dilated renal pelvis, and urinary bladder hypertrophy (Table 8). There were significant increases in the number of fetuses with visceral variations, such as misshapen thymus, ureter abnormality (grade III), and ectopic kidney in the 400-mg/kg/day group. Table 9 reveals the types and incidences of fetal skeletal malformations and variations. Skeletal malformation, such as cleavage ossification of thoracic centrum, was observed in all groups. Although several types of skeletal variations were observed, including incomplete ossification of skull, dumbbell ossification of thoracic centrum, incomplete ossification of thoracic centrum, asymmetric thoracic centrum, supernumerary rib, short rib, and incomplete ossification of sternebra, no significant difference in the number of fetuses with skeletal variations or in the number of affected fetuses was seen between the groups.

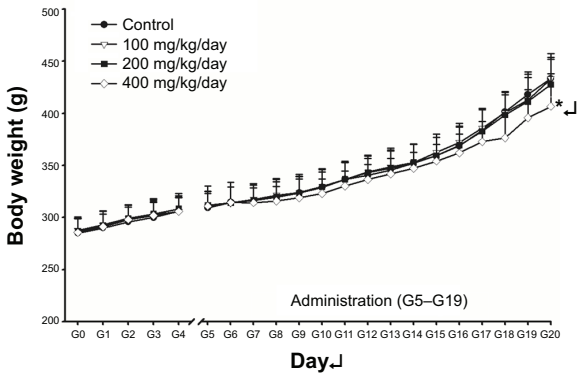

Figure 2 Body weight changes of female rats during the gestation period.
Notes: Pregnant rats were orally treated with $ZnO^{SM20(+)}$ nanoparticles for 15 days (G5–G19) with dose of 100, 200, and 400 mg/kg/day. "G" refers to the number of the days after gestation. "G0" refers to the day on which a rat becomes pregnant. Statistically different from the vehicle control group; *$P<0.05$.

Discussion

Recently, information on the toxicity of ZnO NPs, including liver damage, membrane injury, cytotoxicity, and inflammatory response,[40,41] has been accumulating, but nearly no extensive study on the reproduction and developmental toxicity in mammals was performed. Many studies have been conducted on ZnO NP toxicity, in both environmental species and mammalian cell lines.[42–44] On the other hand, ZnO NPs are used extensively in various commercial applications,[45,46] since nanomaterials have specific physicochemical and electrical properties.[47] As ZnO NPs become a significant source for intended and unintended human exposures, it would be highly desirable and necessary to know more about other possible toxicities of ZnO NPs. This study was conducted to investigate the maternal and developmental toxic potentials of $ZnO^{SM20(+)}$ NPs after

Table 3 Body weights of the pregnant rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Number of pregnant females	23	24	21	23
Gestation day 0	284.9±13.83	287.7±11.68	286.8±13.78	285.5±14.09
Gestation day 1	289.9±13.49	292.9±13.43	292.5±13.33	291.2±12.14
Gestation day 2	295.9±14.27	299.0±13.36	299.0±13.40	298.1±13.76
Gestation day 3	300.0±14.45	302.8±15.03	303.4±13.82	301.9±13.83
Gestation day 4	306.1±14.87	305.7±13.63	308.3±14.68	305.7±13.22
Gestation day 5	309.3±15.61	310.4±12.71	311.6±18.68	311.1±14.13
Gestation day 6	314.5±15.02	313.8±15.55	314.0±15.24	314.3±19.58
Gestation day 7	316.1±15.00	317.4±15.29	316.3±14.71	314.2±14.19
Gestation day 8	319.2±15.24	321.4±16.11	319.6±16.90	315.9±13.88
Gestation day 9	323.5±15.84	324.0±17.25	323.3±16.12	318.9±18.16
Gestation day 10	328.9±14.95	329.7±16.96	329.2±16.85	323.0±14.10
Gestation day 11	336.6±16.16	336.3±16.88	336.6±17.42	330.3±14.41
Gestation day 12	340.5±16.05	343.5±15.62	343.0±16.79	336.4±14.52
Gestation day 13	345.6±18.11	348.6±18.12	347.4±17.24	341.8±15.02
Gestation day 14	352.2±17.94	352.5±17.93	352.8±17.63	347.2±15.22
Gestation day 15	359.3±17.82	362.5±17.63	359.4±17.70	354.2±15.76
Gestation day 16	368.7±18.36	372.1±18.35	369.4±18.22	362.0±18.06
Gestation day 17	383.6±21.07	385.7±18.81	382.7±20.61	372.7±19.66
Gestation day 18	401.6±19.29	400.7±19.25	398.4±21.82	383.9±22.36
Gestation day 19	418.1±21.85	412.9±24.62	411.4±22.89	395.7±26.98
Gestation day 20	433.3±23.90	433.2±20.68	427.6±24.53	406.7±31.27*
Weight gain during pregnancy	148.4±16.58	145.5±14.30	140.8±19.99	121.2±27.23**
Corrected body weight	349.0±21.11	346.8±18.87	342.3±22.09	325.5±26.35*
Gravid uterine weight	84.3±11.63	86.5±7.50	85.3±8.02	81.2±11.30

Notes: Values are expressed as mean ± standard deviation. Statistically different from the vehicle control group; *$P<0.05$, **$P<0.01$.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

oral treatment in SD rats at dose levels of 0, 100, 200, and 400 mg/kg/day from days 5 through 19 of pregnancy. The ZnO NPs were capped with organic ligands, L-serine molecules, which are widely used capping agents for inorganic NPs to enhance the surface charge property.[25–27]

The results showed that a 15-day repeated oral dosing of $ZnO^{SM20(+)}$ NPs during pregnancy resulted in maternal toxicity at 400 mg/kg/day, but the same dose did not cause serious teratogenic toxicity. Although salivation was observed in all treated groups, it was not considered to be related to the

Table 4 Food consumptions of the pregnant rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Number of pregnant females	23	24	21	23
Gestation day 0	22.56±3.59	23.69±3.53	23.00±2.51	23.77±8.43
Gestation day 2	27.04±3.00	26.41±3.65	27.64±2.48	29.07±7.40
Gestation day 4	28.53±6.45	26.77±4.08	27.25±2.72	29.76±10.89
Gestation day 6	26.21±3.85	25.55±2.67	24.93±3.86	23.03±4.00
Gestation day 8	28.40±7.89	26.75±3.59	27.11±3.80	27.06±4.73
Gestation day 10	29.19±6.28	27.11±5.29	28.22±4.97	25.83±3.50
Gestation day 12	28.19±3.01	28.59±3.26	27.97±3.55	27.33±3.40
Gestation day 14	28.54±2.84	27.93±3.18	26.90±3.85	27.56±5.55
Gestation day 16	31.38±3.90	30.35±4.27	29.43±5.79	28.39±6.82
Gestation day 18	31.55±3.60	29.39±4.84	26.21±4.17*	25.87±4.75*

Notes: Values are expressed as mean ± standard deviation. Statistically different from the vehicle control group; *$P<0.05$.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Table 5 Absolute and relative organ weights of pregnant rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	Unit	$ZnO^{SM20(+)}$ NPs			
		Vehicle(Serine)	100	200	400
Number of pregnant females		23	24	21	23
Body weight at term (g)		433.3±23.90	433.2±20.68	427.6±24.53	406.7±31.27
Liver	(g)	15.63±1.46	15.85±1.16	15.09±1.22	14.22±1.40*
	(g%)	3.60±0.22	3.66±0.20	3.53±0.20	3.50±0.29
Kidney, left	(g)	1.07±0.07	1.07±0.07	1.05±0.08	1.05±0.08
	(g%)	0.25±0.02	0.25±0.02	0.25±0.02	0.26±0.03
Kidney, right	(g)	1.11±0.07	1.08±0.07	1.06±0.09	1.07±0.09
	(g%)	0.26±0.02	0.25±0.02	0.25±0.02	0.26±0.03
Spleen	(g)	0.68±0.15	0.61±0.16	0.72±0.16	0.63±0.13
	(g%)	0.16±0.03	0.14±0.04	0.17±0.04	0.15±0.04
Adrenal gland, left	(g)	0.043±0.008	0.049±0.010	0.046±0.010	0.050±0.010
	(g%)	0.010±0.002	0.011±0.003	0.011±0.002	0.012±0.003*
Adrenal gland, right	(g)	0.041±0.008	0.044±0.010	0.045±0.007	0.051±0.014*
	(g%)	0.009±0.002	0.010±0.002	0.011±0.002	0.013±0.005**
Ovary, left	(g)	0.065±0.013	0.071±0.019	0.073±0.015	0.064±0.016
	(g%)	0.015±0.003	0.016±0.004	0.017±0.004	0.016±0.005
Ovary, right	(g)	0.066±0.014	0.064±0.017	0.067±0.012	0.071±0.014
	(g%)	0.015±0.003	0.015±0.004	0.016±0.003	0.018±0.004
Brain	(g)	2.05±0.18	2.01±0.07	2.00±0.08	1.96±0.13
	(g%)	0.47±0.05	0.46±0.02	0.47±0.03	0.48±0.03
Pituitary gland	(g)	0.015±0.004	0.015±0.003	0.016±0.003	0.014±0.004
	(g%)	0.003±0.001	0.004±0.001	0.004±0.001	0.003±0.001
Lung	(g)	1.36±0.11	1.35±0.10	1.39±0.10	1.32±0.14
	(g%)	0.31±0.02	0.31±0.02	0.32±0.02	0.33±0.04
Heart	(g)	1.17±0.11	1.16±0.12	1.16±0.12	1.09±0.12
	(g%)	0.27±0.03	0.27±0.02	0.27±0.03	0.27±0.02
Uterus (gravid)	(g)	84.29±11.63	86.45±7.50	85.26±8.02	81.18±11.30
	(g%)	19.44±2.36	19.97±1.62	19.95±1.73	19.95±2.32

Notes: Values are expressed as mean ± standard deviation (g). Statistically different from the vehicle control group; *$P<0.05$, **$P<0.01$.
Abbreviations: bw, body weight; g%, relative weight of organ (g) to body weight (g); NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Table 6 Cesarean section data from pregnant rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Number of dams	23	24	21	23
Number of corpora lutea	19.3±3.66	18.3±2.94	19.2±4.58	18.5±2.11
Number of implantation sites	15.3±1.57	15.9±1.35	15.6±1.43	16.1±1.76
Implantation rate (%)	81.0±13.96	88.6±13.48	84.0±13.27	87.6±9.35
Fetal deaths	0.6±1.12	0.6±0.78	0.9±0.85	1.3±3.30
Resorption : early	0.5±1.08	0.3±0.53	0.5±0.60	0.3±0.65
: late	0.0±0.21	0.3±0.55	0.3±0.46	0.2±0.42
Dead fetuses	0.0±0.00	0.0±0.20	0.1±0.44	0.7±3.33
Litter size	14.7±1.64	15.3±1.34	14.8±1.67	15.5±1.95
Male/female	179/159	177/191	169/141	173/167
Sex ratio	1.13	0.93	1.20	1.04
Fetal weight (g) : male	4.03±0.30	3.96±0.22	4.07±0.21	3.71±0.29**
: female	3.85±0.35	3.78±0.22	3.87±0.21	3.57±0.29*
Placental weight (g)	0.56±0.13	0.56±0.05	0.56±0.07	0.54±0.06

Notes: Values are expressed as mean ± standard deviation. Statistically different from the vehicle control group; *$P<0.05$, **$P<0.01$.
Abbreviations: bw, body weight; g%, relative weight of organ (g) to body weight (g); NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Table 7 External alterations in fetuses from pregnant rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Litters examined	23	24	21	23
External examination				
Fetuses examined	338	368	310	340
Fetuses with malformations (%)[a]	0	0	0	0
Litters affected (%)[b]	0	0	0	0
Fetuses with variations, n (%)[a]	4 (1.18)	6 (1.63)	1 (0.32)	7 (2.06)
Litters affected, n (%)[b]	4 (17.39)	6 (25.00)	1 (4.76)	7 (30.43)
Hematoma	4	6	1	7

Notes: [a]A single fetus may be represented more than once in listing individual defects. [b]Includes litters with one or more affected fetuses.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

$ZnO^{SM20(+)}$ NP treatment because the salivation was observed sporadically and was not dose dependent. This finding may attribute to irritation or stress of the test subjects. $ZnO^{SM20(+)}$ NPs induced significant maternal toxicity in the 400-mg/kg/day group, which was evidenced by suppressed body-weight gain, decreased liver weight, and increased adrenal gland weights. The suppressed body-weight gain in the high-dose group could have been a direct effect of $ZnO^{SM20(+)}$ NPs on pregnant dams, because the gravid uterine weight of the 400-mg/kg/day group at term was similar to that of the control group.

In general reproductive toxicity studies, it is well known that body and organ weights could be sensitive indicators of potentially toxic chemicals.[48–50] The significant decreases in the absolute liver weight in 400-mg/kg/day group were attributed to the administration of $ZnO^{SM20(+)}$ NPs, since these changes were remarkable in comparison with the control group, showing a clear-cut dose–response relationship. Earlier studies revealed that after uptake in the gastrointestinal tract, the biodistribution of engineered NPs was located at the liver and kidney.[15,51,52] Significant weight increases of the adrenal glands were observed in the 400-mg/kg/day group, which could be considered as a treatment-related effect, since these changes was remarkably distinguishable in comparison with the control group. Previous studies demonstrated that the function and weight of adrenal glands is adversely effected by various stressful factors.[53–56] So the increased weights of adrenal glands are also considered to be related to stress responses, induced by the administration of $ZnO^{SM20(+)}$ NPs, which is consistent with the decreased body-weight gain in the group.

Up to now, several studies have evaluated the toxic effects of ZnO nanomaterials in vitro and in vivo. Yang et al[57] demonstrated with primary mouse embryo fibroblast cells that the interrelationship existed between particle size, shape, chemical composition, and toxicology effects of carbon black, single-wall carbon nanotube, silicon dioxide, and ZnO NPs.

Table 8 Visceral alterations in fetuses from rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Litters examined	23	24	21	23
Visceral examination				
Fetuses examined	169	180	154	165
Fetuses with malformations (%)	0	0	0	0
Litters affected (%)	0	0	0	0
Fetuses with variations, n (%)[a]	71 (42.01)	85 (47.22)	70 (45.45)	69 (41.82)
Litters affected, n (%)[b]	19 (82.61)	24 (100)	21 (100)	20 (86.96)
Misshapen thymus	11	12	12	32**
Ureter abnormality				
Grade III	9	14	8	20*
Dilated renal pelvis	0	0	0	2
Large kidney	0	0	0	3

Notes: [a]A single fetus may be represented more than once in listing individual defects. [b]Includes litters with one or more affected fetuses. Statistically different from the vehicle control group; *$P<0.05$, **$P<0.01$.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Table 9 Skeletal variations in fetuses from rats treated with $ZnO^{SM20(+)}$ NPs

Dose (mg/kg bw/day)	$ZnO^{SM20(+)}$ NPs			
	Vehicle(Serine)	100	200	400
Litters examined	23	24	21	23
Skeletal examination				
Fetuses examined	169	188	156	175
Fetuses with malformations, n (%)[a]	2 (1.18)	3 (1.60)	2 (1.28)	3 (1.71)
Litters affected, n (%)[b]	2 (8.70)	3 (12.50)	2 (9.52)	3 (13.04)
Thoracic centrum				
Cleavage ossification	2	3	2	3
Fetuses with variations, n (%)[a]	16 (9.47)	22 (11.70)	6 (3.85)	17 (9.71)
Litters affected, n (%)[b]	11 (47.83)	13 (54.17)	5 (23.81)	9 (39.13)
Skull				
Incomplete ossification	0	0	0	1
Thoracic centrum				
Dumbbell ossification	4	3	1	1
Asymmetric	1	2	1	0
Incomplete ossification	4	12	3	4
Rib				
Short rib	0	0	0	1
Supernumerary	5	5	1	6
Sternebra				
Incomplete ossification	2	0	0	4

Notes: [a]A single fetus may be represented more than once in listing individual defects. [b]Includes litters with one or more affected fetuses.
Abbreviations: bw, body weight; NPs, nanoparticles; $ZnO^{SM20(+)}$, 20-nm positively charged ZnO.

Xia et al[58] reported that ZnO induced toxicity in RAW 264.7 cell lines, leading to the generation of reactive oxygen species, oxidant injury, excitation of inflammation, and cell death. According to the bacterial toxicity study of ZnO NPs,[59] ZnO NPs damaged bactericidal wall by increasing membrane permeability. In nematodes,[60] the toxicity of manufactured ZnO NPs (1.5 nm) might have caused the intracellular biotransformation. Interestingly, comparable toxicities of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ were observed with algae.[61]

External and skeletal variations were found in few fetuses (Tables 7–9), as to morphological variation or malformation findings from the fetuses of $ZnO^{SM20(+)}$ NP-treated dams. In particular, significant increases in the number of fetuses with visceral variations were observed in the 400 mg/kg of $ZnO^{SM20(+)}$ NPs group, which could be considered as a treatment-related effect, since these changes were remarkable in comparison with the control group. Significant increases in the number of fetuses with visceral variations were observed in the 400 mg/kg/day of $ZnO^{SM20(+)}$ NPs group, which may have not been a direct influence of the test substance. Increases in visceral variations (misshapen thymus and ureter abnormality) were also considered to be indirect effects of maternal toxicity or a spontaneous effect, because these changes were not significant, and the treatment-related fetal malformation was not found at all tested doses. The observed fetal variations in the present study were not considered to be caused by the administration of $ZnO^{SM20(+)}$ NPs since they occurred at a very low rate without exhibiting a dose–response relationship, or they were sporadically observed in fetuses from normal control rats.[62–64]

The concentration of Zn in the fetuses in the vehicle and $ZnO^{SM20(+)}$ NP-dose groups are shown in Figure 1. Different distributions of NPs ingested into the body could be found at different regions due to their small size. After the exposure of 400 mg/kg/day of $ZnO^{SM20(+)}$ NPs, a significant increase of Zn contents was not found in the $ZnO^{SM20(+)}$ NP-treated rats in comparison with the control group. Although the increases in Zn concentrations in the exposed fetuses exhibited a trend, their influence on intrauterine fetal growth/development could be considered as weak effects.

In the present study, effects of $ZnO^{SM20(+)}$ NPs on intrauterine growth and on fetal visceral morphology were observed. Lower mean maternal body weights and body-weight gain in comparison to the control group were observed in the group treated with 400 mg/kg/day $ZnO^{SM20(+)}$ NPs in a dose-related manner. Lower mean corrected body weight was noted in this group at necropsy. Furthermore, the reduction in body-weight gain during the late gestation period in the 400-mg/kg/day group was considered to be mainly due to the growth of fetuses, since the fetal weight was markedly decreased in this group. Similarly, decreased body-weight gain during the late gestation period was due to the decreased food consumption,

which resulted in reduction of fetal weight in utero. On the contrary, some positive effects of other NPs were noted in similar reproduction/developmental toxicity studies. When silica and titanium dioxide NPs were administered to pregnant mice intravenously, pregnancy complications were observed. Mice also had smaller uteri and smaller fetuses than untreated controls.[65] Also, when silicon crystal NPs (50 mg/kg) were injected, these NPs led to reduction in body-weight gain in pregnant rats and newborn rats at different stages of the experiment, but without noticeable effect on other parameters of physical development of rat progeny or teratogenic effects.[66] In addition, oral administrations of silver NPs (250 mg/kg) caused a relatively low toxic effect.[67]

Unpublished 90-day repeated oral-dose and genotoxicity data from our previous studies are briefly summarized, as follows. The ZnO NPs did not cause any significant changes at the endpoints of repeated-dose toxicity, including clinical observation, functional observation, body-weight gain, water and food consumption, urinalysis, hematology, serum biochemistry, organ weight, toxicokinetic study, tissue distribution, and histopathology in the repeated study. In addition, when 14-day recovery groups were set for the high-dose groups in both sexes, no significant result was observed in functional examination, body-weight gain, water and food consumption, urinalysis, necropsy, and organ weight. Hence, it was concluded that no significant induced effect was affected by the treatment of the ZnO NPs. However, dose-dependent depositions of ZnO NPs were also found in the pancreas, glandular stomach, and eyes, indicating potential systemic distributions of ZnO NPs in the mammalian tissues. Furthermore, genotoxicity tests of ZnO NPs did not show any gene mutation potential in vitro, chromosome aberration assay in vitro, or micronucleus test in vivo. Moreover, in vivo micronucleus test and in vivo comet assay in the 90-day study also indicated negative results, supporting that ZnO NPs did not cause any DNA damage.

Conclusion

In summary, prenatal and developmental toxicity of positively charged ZnO NPs were tested in accordance with the OECD test guideline 414 and GLP principle. Pregnant female rats were orally treated with $ZnO^{SM20(+)}$ NPs, from GD 5–19, with doses of 100, 200, and 400 mg/kg/day. The results of this developmental toxicity study suggest that administration of $ZnO^{SM20(+)}$ NPs to pregnant rats had minimal impact on intrauterine fetal growth and development, even in the high dose of 400 mg/kg/day. Based on the results of these studies, a dosage level of 200 mg/kg/day of $ZnO^{SM20(+)}$ NPs was considered the no-observed-adverse-effect level for both maternal toxicity and embryo–fetal development. Although the result of the current study clearly showed the adverse effects of $ZnO^{SM20(+)}$ NPs on pregnant rats and fetuses, information on the effects of ZnO NPs on reproduction/development are not sufficient at this time.

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Disclosure

The authors report no conflicts of interest in this work.

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