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Effects of Platinum Nanoparticles on the Postnatal Development of Mouse Pups by Maternal Exposure

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ABSTRACT

Objectives : Platinum nanoparticles (PNPs) are potentially useful for sensing, catalysis, and other applications in the biological and medical sciences. However, little is known about PNP toxicity. In this study, adverse effects of PNPs on the postnatal development of mouse pubs were investigated.
Methods : PNPs (size: 20 nm) were prepared and orally administered to mice during premating, gestation, and lactation periods (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg). Maternal and pup toxicity were evaluated.
Results : PNPs did not affect blood biochemical parameters or mortality in dams during the experimental period. Histopathological signs were not observed and pup number was not different between the control and treated groups. Deformity and stillbirth were not observed in the pups. However, PNPs increased pup mortality and decreased the infant growth rate during the lactation period.
Conclusion : PNPs may have adverse effects to the postnatal development of mouse pups.

Key words : Growth rate, Infant mortality, Mice, Platinum nanoparticles, Reproductive toxicity

INTRODUCTION

Metal nanoparticles such as titanium, silver, and platinum have potentially useful applications in many industrial fields. Among these metal nanoparticles, platinum nanoparticles (PNPs) have been widely used as a catalyst due to their high conductance and reactivity [1,2]. PNPs have also been shown to have a protective effect on reactive oxygen species. In previous reports, PNPs do not show cytotoxicity in several different cultured cells (TIG-1, MI-38, MRC-5, Hela, and HepG2), although cellular uptake of PNPs does occur in a time- and dose-dependent manner [3], PNPs have been shown to protect cells from oxidation-induced inflammation by scavenging superoxide anions ($O_2 \cdot -$) and hydroxyl radicals ($\cdot OH$) from aqueous solutions, and they inhibit pulmonary inflammation in mice exposed to cigarette smoke by preventing antioxidant depletion and inhibiting neutrophil infiltration and NF-κB activation [4]. However, some workers who have been exposed to platinum during the refining processes manifested inflammatory diseases such as rhinitis, conjunctivitis, and asthma. Inhalation is the most common route of allergen entry, and 'platinosis' is considered to be an allergic response to complex platinum salts. Furthermore, some researchers suggest that platinum induces damage to cells through oxidative stress and inflammatory signals [5,6]. Although this topic has prompted lively debate, very little infor-

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mation is available regarding the safety and potential hazards of manufactured PNPs.

In this study, PNPs were prepared from potassium hexachloroplatinate (K_2PtCl_6) and were applied to both male and female mice. Blood biochemistry, birth rate, post-natal growth, development, and mortality of pups were observed in mice treated with PNPs (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg) during pre-mating, gestation, and lactation periods by oral administration.

MATERIALS AND METHODS

1. Manufacture of PNPs

A mixture of 3 mL of 6 mM K_2PtCl_6 aqueous solution, x mL of double-distilled water, and y mL of alcohol (x+y=197 mL) containing 100 mg of PVP (polyvinylpyrrolidone) was refluxed into a 250 mL flask for 3 h in an air-conditioned environment until the ethanol evaporated. The resulting product in the water-phase showed a black suspension. A solvent control was prepared by the same procedure without K_2PtCl_6 [7]. Particle size measurements were then performed using a submicron particle sizer (NICOMPTM, CA, USA), and images of the nanoparticles were acquired by transmission electron microscopy (TEM; JEM1010, JEOL, Japan). The average particle size was 20.9±11.4 nm (Fig. 1).

2. Animals

ICR male and female mice (6 weeks old) were purchased from Orient-Bio Animal Company (Seongnam, Korea) and were acclimated to the animal room conditions prior to the initiation of the study. The environmental conditions were strictly controlled with a temperature of 23±1°C, relative humidity of 55±5%, and a 12 h light/dark cycle. All the animals used in this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals" issued by the Animal Care and Use Committee of National Veterinary Research and Quarantine Service (NVRQS) For mating, one male and one female mouse were kept in the same cage. After the gestation, the male mouse was removed. Total number of female mice in each group was 11. Low, medium and high dosage groups were treated with PNPs only for female mice.

3. Treatment and sample preparation for analysis

PNPs were repeatedly administrated orally to both male and female mice in doses of 0.25 mg/kg (low),

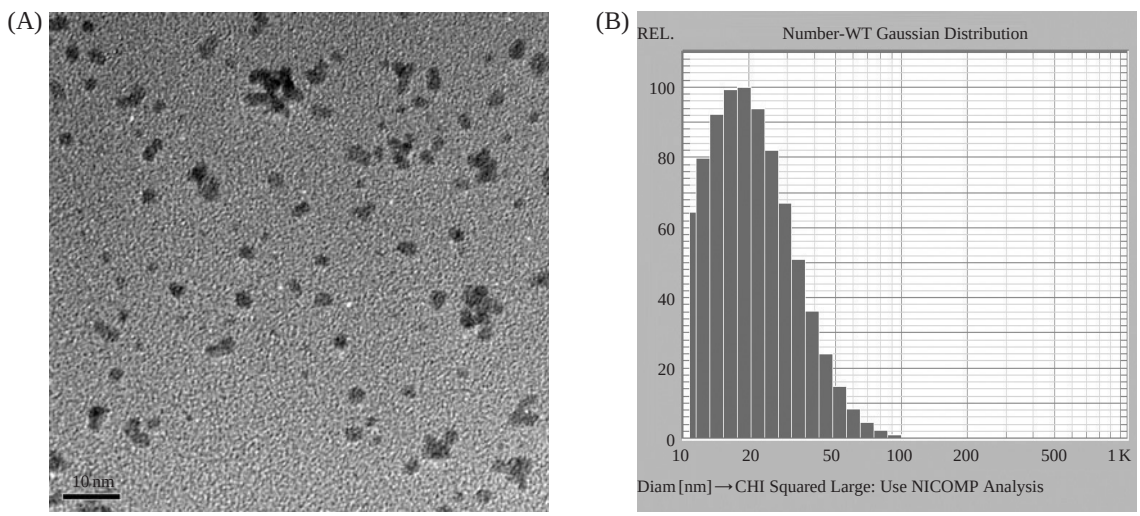

Fig. 1. TEM (transmission electron microscopy) image and size distribution of PNPs.
(A) TEM image, (B) Size distribution.

0.5 mg/kg (medium), and 1 mg/kg (high) for 14 days before mating. Then mating was done by keeping one male and one female mouse in a cage. After gestation was confirmed, the male mouse was removed and oral administration to female mice was continued until delivery and for 4 days after the delivery. The control group was treated with vehicles used in the preparation of PNPs. By preliminary screening of lethal dose of PNPs, lower level of non-lethal dose was tested in this study. The body weights of the mothers were measured on a weekly basis. Body weights of pups were also measured. Diet and water consumption were measured 2 times per week.

4. Maternal evaluation

At 5 days after delivery, blood was collected from the retro-orbital venous plexus of the female mother mice using heparinized capillary tubes and centrifuged at 3,000 rpm for 10 min to make serum for biochemical analysis. The sera were stored in the −80˚C freezer prior to analysis. Total protein, albumin, AST (aspartate aminotransferase), ALT (Alanine aminotransferase), ALP (alkaline phosphatase), creatinine, BUN (blood urea nitrogen), γ-GTP (glutamyl transpeptidase), and total cholesterol were measured using an autoanalyzer (Hitachi7180, Hitachi, Japan). After blood collection from the retro-orbital venous plexus, samplings of the kidneys, lungs, and livers were done to measure platinum accumulation. Same weight of tissue samples were pooled from 3 to 4 mice (sample number, n=3), frozen in a −80˚C freezer, and dehydrated with a freeze-dryer (Ilshin Co., Yangju, Korea). The mixed dried tissues (about 200 mg) were digested in a solution of 7 mL of 70% HNO_3 and 1 mL of 30% H_2O_2 using the microwave digestion system (Milestone, Sorisole, Italy) for the determination of platinum accumulation. The concentration of platinum in the lysate which was diluted by distilled deioniazed water, was analyzed with ICP-MS (Elan6100/Perkin Elmer, USA) in the Korean Basic Science Institute (KBSI, Seoul, Korea). The detection limit in the final sample was 0.001 ppb.

Histopathology analysis was performed under the responsibility of a pathologist in Biotoxtech (Cheongwon-gun, Chungbuk, Korea), one of the Good Laboratory Practice (GLP) institutes of Korea. Livers as well as reproductive organs including ovaries and uteruses of the control and treated female mice were fixed with 10% neutral buffered formalin at 5 days after delivery and processed by routine histological techniques. After paraffin embedding, 3 μm sections were cut and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

The concentrations of cytokines, including TFN-α, in the serum were determined using commercially available ELISA kits (eBioscience, San Diego, CA, USA). Briefly, each well of a microplate was coated with 100 μL of capture antibody and incubated overnight at 4˚C. After washing and blocking with assay dilution, serum or standard materials were added to the individual wells; then the plates were maintained for 2 h at room temperature. The plates were then washed by washing buffer, biotin-conjugated detecting mouse antibody was added to each well, and they were incubated at room temperature for 1 h. The plates were washed again and further incubated with avidin-HRP for 30 min before detection with TMB solution. Finally, reactions were stopped by adding 1 M H_3PO_4, and the absorbance at 450 nm was measured with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amount of cytokine was calculated from the linear portion of the generated standard curve.

5. Pup evaluation

Naturally delivered pups per dam were counted and evaluated for clinical signs, size, and body weight. Pups were also examined for external malformations or variation. Deaths of pups were also counted.

6. Statistical analysis

The results obtained from the chemically treated groups were compared to those of the control group. The values were compared using the one-way ANOVA test. When statistically different groups were observed, the Dunnett test was done to determine the significance

Table 1. Blood biochemical parameters in mother mice after oral administration of PNPs (n=11)

	Control	0.25 mg/kg	0.50 mg/kg	1.00 mg/kg
TP	4.48±0.70	4.40±0.39	4.42±0.49	4.88±0.13
ALB	2.78±0.37	2.82±0.23	2.86±0.27	3.14±0.09
AST	80.00±23.70	72.60±16.20	48.80±6.22	64.00±21.41
ALT	42.40±13.72	41.40±9.40	33.00±5.52	33.80±1.64
ALP	67.80±15.07	65.00±5.24	64.60±15.36	61.60±8.88
Creatinine	0.42±0.04	0.44±0.05	0.40±0.00	0.42±0.04
BUN	18.60±4.01	19.74±3.27	18.52±3.77	16.22±1.13
r-GTP	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Chol	87.60±10.83	89.60±11.80	97.00±27.03	93.60±22.17

TP: total protein, ALB: Albumin, AST: aspartate aminotransferas, ALT: alanine aminotransferase, ALP: alkaline phosphatase, BUN: blood urea nitrogen, GTP: glutamyl transpeptidase, Chol: cholesterol.

among the control group and the treated groups.

RESULTS

1. Maternal evaluations

There were no differences in diet and water consumption between the control and treated groups (0.25 mg/kg, 0.50 mg/kg, and 1.00 mg/kg PNPs) during the premating, gestation, and post-delivery periods (number of dams=11). Specific behavioral activity different from the control group was not seen in the treated groups. During the pre-mating period, daily diet consumption was about 4-5 g per mouse, and water consumption was about 4-5 mL per mouse. No dead dams were found during the experimental periods due to the treatment of PNPs. Female dam mice treated with PNPs were sacrificed (n=11) at 5 days after delivery and blood chemistry analysis was performed. As shown in Table 1, no biochemical parameters were affected by PNPs-treatment.

When platinum was measured in the tissues of mother mice sacrificed at 5 days after delivery, it was detected only in the lungs. The concentrations of platinum were 2.91±4.34 ng/g lung tissue in the 0.5 mg/kg PNPs-treated group and 14.33±16.41 ng/g lung tissue in the 1 mg/kg PNPs-treated group. In the liver and kidneys, platinum was not detected (Table 2).

Histopathological changes were examined in the livers, ovaries, and uteruses of the dams treated with 1 mg/kg PNPs. No abnormalities were found in either the control or the treated groups (data not shown). However, TNF-α in the serum was significantly elevated in dams treated with PNPs. TNF-α level was increased to 6.93±0.06 pg/mL in the 0.25 mg/kg-treated group, 6.89±0.02 pg/mL in the 0.5 mg/kg-treated group, and 7.45±0.03 pg/mL in the 1.0 mg/kg-treated group, while the level of the control group was 2.22±0.00 pg/mL. IFN-γ was not elevated by PNPs-treatment and TGF-β was not detected in any of the groups (Fig. 2).

Table 2. Accumulation of platinum in the organs of mother mice after oral administration of PNPs (pooling tissues, n=3) (ng/g)

	Liver	Lung	Kidney	Fetus
Control	ND	ND	ND	ND
0.25 mg/kg	ND	ND	ND	ND
0.50 mg/kg	ND	2.91±4.34	ND	ND
1.00 mg/kg	ND	14.33±16.41	ND	ND

ND: not detect.

2. Pup evaluations

As shown in Table 3, there are no statistically significant differences in the number of pups among the control and the PNPs-treated groups. No dead fetuses were found during the gestation period in either the control or the PNPs-treated groups. The average number of pups from a dam was 12.6±4.5 in the 0.25 mg/kg-treated group, 14±1.9 in the 0.5 mg/kg-treated

Table 3. Evaluation of dams and pubs after oral administration of PNPs

		Control group	Treated group		
			0.25 mg/kg	0.5 mg/kg	1.0 mg/kg
Dam	Mortality	None	None	None	None
	Behavioral defect	None	None	None	None
	Histopathology (liver, ovary, uterus)	None	None	None	None
	Delivered pub number	11±4.1	12.6±4.5	14±1.9	13.2±3
	Dams to show pub mortality	2	2	4	5
Pub	Dead fetus	None	None	None	None
	Total dead pubs within 4 days after birth	2	6	6	8
	Weight gain within 4 days after birth (g)	1.27±0.09	1.07±0.09	1.10±0.04	1.02±0.06

(Female mice in each group was 11)

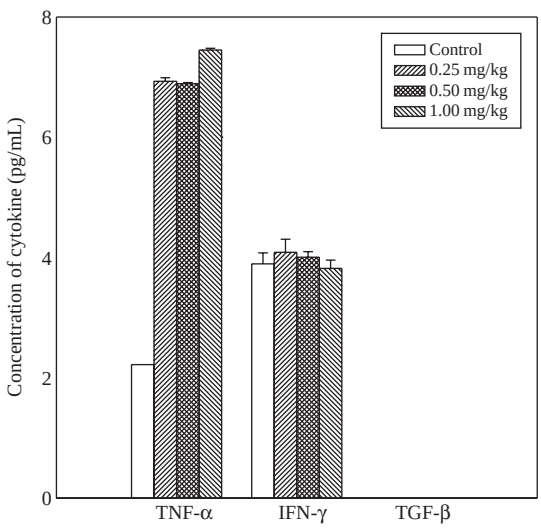

Fig. 2. Serum levels of cytokines in mother mice after oral adaministration of PNPs (pooling sera, n=3).

group, 13.2±3 in the 1.0 mg/kg-treated group, and 11±4.1 in the control group.

No deformities in the pups were found in either the control or PNPs-treated groups. When the platinum level was determined in the whole body of the pups, platinum was not detected in the treated groups, which suggested a possibility that PNPs do not penetrate the placenta-barrier. However, the mortality of pups after delivery in the treated groups was increased. Table 3 shows the number of dead pups during the lactation period. Within 4 days after delivery, 6 pups in the 0.25 mg/kg-treated group, 6 pups in the 0.5 mg/kg-treated group, and 8 pups in the 1 mg/kg-treated group were found dead, while 2 dead pups were observed in the control group. Weight gain of pups at 4 days after delivery was also decreased in pups from the PNPs-treated groups. As shown in Table 3, body weight of pups was 1.07±0.09 g in the 0.25 mg/kg-treated group, 1.10±0.04 g in 0.5 mg/kg-treated group, and 1.02±0.06 g in the 1 mg/kg-treated group, while the body weight of the pups in the control group was 1.27±0.09 g.

DISCUSSION

With the increase in the applications for nanoparticles both in industrial fields and in consumer products, toxicity tests of nanoparticles have been widely performed [8,9]. It has been accepted by many toxicologists that three key elements of the toxicity screening strategy are the identification of physicochemical characteristics, in vitro assays, and in vivo assays using animals. Regarding the in vivo tests, repeated dose toxicity tests with different exposure pathways, mutagenicity, and toxicokinetics seem to be focused on the toxicity tests [10-13]. However, the potential effects of nanoparticles on the reproductive system, placenta translocation, and fetus development are still far from even basic evaluations, although some researchers suggested the importance of reproductive toxicity of nanoparticles [14-16]. For example, C60

intraperitoneally administered to pregnant mice was distributed into the yolk sac and embryos and had a harmful effect on both concepts [17]. Carbon black nanoparticles intratracheally administered also induced adverse effects on the mouse male reproductive function [14,18] suggested that AgNPs induced heat shock stress, oxidative stress, DNA damage, and apoptosis in D. melanogaster and thus warrants more careful assessment of AgNPs using in vivo models to determine if chronic exposure triggers developmental and reproductive toxicity [14]. Many researchers also suggested that maternal exposure to air pollutants during pregnancy might have adverse effects including preterm birth, low birth rate, intrauterine growth restriction, birth defects, and intrauterine and infant mortality on the developing fetus [19].

In this study, PVP-coated PNPs were prepared and the toxicity on dams and pups was evaluated after oral administration. No dead dams or fetuses were found after oral treatment. PNPs were confirmed to be absorbed into the bloodstream after oral administration based on the distribution in the lungs. Although bioavailability was not evaluated in this study, a portion of the administered PNPs seemed to be absorbed through the gastrointestinal tract. Among the tissues, the lung was the main target of PNPs. PNPs were not accumulated in the liver or kidneys (Table 2). As shown in Table 2, the analytical result was not fine but the standard deviation is very large. This is due to the relative low level of PNPs in organs and the sample number is very small (n=3). Although the result was not refined in this study, we suggest a possibility to the accumulation of PNPs in lung but not in fetus, which means no PNPs translocation through placenta.

Liver toxicity, evaluated by serum AST, ALT, and ALP and albumin level, was not shown and this seemed to be due to the low distribution of PNPs in the liver. Neither BUN nor creatinine levels were affected by PNPs; this suggested that kidney function may not be disturbed (Table 1). In a previous study, it was reported that when mice were repeatedly exposed for 28 days by oral administration with 30 mg/kg, 300 mg/kg, or 1,000 mg/kg dose of silver nanoparticles (60 nm) in carboxyl-methyl cellulose (CMC), silver nanoparticles did not induce any significant changes in body weight. However, alkaline phosphatase (ALP) and cholesterol values were significantly increased in the blood of the groups treated with more than 300 mg/kg AgNPs. The accumulation in tissues was observed to be dose-dependent, but genetic toxicity-related reactions were not shown in all groups [20].

When PNPs were applied to pregnant mice, the PNPs were not detected in the pups. This suggests that PNPs may not translocate the placental-barrier. If the dam's dosage was too low to reach a high enough blood level, or if the bioavailability of PNPs was too low after oral administration, the PNPs may not be detected in the pups. This may be another reason why PNPs were not detected in pups. Further study will be needed.

No difference in birthrate was found in either the PNPs-treated or control groups (Table 3). However, the number of dead pups during the lactation period was increased 3 fold in the 0.25 mg/kg and 0.5 mg/kg PNPs-treated groups, and 4 fold in the 1 mg/kg PNPs-treated group comparing with the control group. There were 2 dams that showed pup mortality in the control group, 2 in the 0.25 mg/kg-treated group, 4 in the 0.5 mg/kg-treated group, and 5 in the 1.0 mg/kg-treated group.

No reports have been released on the increase of pup mortality due to nanoparticles until now. We do not know the exact mechanisms of PNP-induced mortality in pups at the present and further study will be necessary. The growth rates in PNP-treated groups were decreased to 84.3% (low dose), 86.6% (medium dose), and 80.3% (high dose) compared with that of the control group. Pups with low body weight and low activity may not be able to compete to get the milk from the mother. This seemed to cause malnutrition and to lead to death.

TNF-α plays physiologically and pathologically important roles in the placenta and endometrium and controls diverse cellular functions such as apoptosis, inflammation, sepsis, and the development of the immune system [21]. Pregnancy was associated with

a suppression of cytokine responses, in particular of the pro-inflammatory cytokines IFN-γ and TNF-α [22]. The expression of TNF-α is regulated by ROS and TNF-α in turn regulates ROS production [23,24]. In this study, TNF-α was increased about 3 fold in mother mice by PNPs, whereas IFN-γ remained at a similar level in all the groups. This may be another signal to trigger the undesirable physiology in pups to cause the increase in mortality.

In summary, PNPs did not affect blood biochemical parameters or mortality in dams after oral administration during the pre-mating, gestation, and post-delivery periods. Birth rates were not different among the control and treated groups. Deformity of pups and stillbirths were not observed after treatment with PNPs. However, PNPs increased pup mortality and decreased the infant growth rate during the lactation period.

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