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Deleterious effects in reproduction and developmental immunity elicited by pulmonary iron oxide nanoparticles

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ABSTRACT

With the extensive application of iron oxide nanoparticles (FeNPs), attention about their potential risks to human health is also rapidly raising, particularly in sensitive subgroups such as pregnant women and babies. In this study, we a single instilled intratracheally FeNPs (1, 2, and 4 mg/kg) to the male and female parent mice, mated, then assessed reproductive toxicity according to the modified OECD TG 421. During the pre-mating period (14 days), two female parent mice died at 4 mg/kg dose, and the body weight gain dose-dependently decreased in male and female parent mice exposed to FeNPs. Additionally, iron accumulation and the enhanced expression of MHC class II molecules were observed in the ovary and the testis of parent mice exposed to the highest dose of FeNPs, and the total sex ratio (male/female) of the offspring mice increased in the groups exposed to FeNPs. Following, we a single instilled intratracheally to their offspring mice with the same doses and evaluated the immunotoxic response on day 28. The increased mortality and significant hematological- and biochemical- changes were observed in offspring mice exposed at 4 mg/kg dose, especially in female mice. More interestingly, balance of the immune response was shifted to a different direction in male and female offspring mice. Taken together, we conclude that the NOAEL for reproductive and developmental toxicity of FeNPs may be lower than 2 mg/kg, and that female mice may show more sensitive response to FeNPs exposure than male mice. Furthermore, we suggest that further studies are necessary to identify causes of both the alteration in sex ratio of offspring mice and different immune response in male and female offspring mice.

1. Introduction

Iron, a representative particulate material-bound heavy metal, has been suggested as an important cause that provokes the respiratory symptoms by forming reactive oxygen species (ROS) when we inhaled ambient particles (Aust et al., 2002; Jacobs et al., 2012; Wang et al., 2014). Meanwhile, iron oxide nanoparticles (FeNPs) have been widely studied with the great potential for revolutionizing applications, such as drug delivery, magnetic resonance imaging agents, soil and groundwater remediation, and as photocatalysts (Liu, 2006; Penn et al., 2003). Therefore, it is anticipated that the level in the environment and human exposure to FeNPs may notably increase over the coming decade, enhancing the potential risks to human health, particularly in sensitive subgroups such as pregnant women and babies.

Nanoparticles (NPs) can easily penetrate through biological membranes due to their small size (Chu et al., 2010; Guarnieri et al., 2014; Lee et al., 2013; Schädlich et al., 2012; Wang et al., 2013). NPs also have an increased surface area ratio to mass compared to the micro-sized particles of the same substance, thus their chemical/catalytic reactivity are markedly enhanced. For example, carboxyl-coated FeNPs (10, 20, 30, and 40 nm) is primarily distributed in the liver and the spleen, the smallest size (10 nm) penetrated more readily into the brain and the uterus than other sizes, and smaller FeNPs (10 and 20 nm) effectively altered the expression level of oxidative stress-, iron transport-, metabolism-, and apoptosis-related genes (Yang et al., 2015). In addition, growing evidences suggest that exposure to harmful environmental particles during pregnancy period can cause adverse health effects on the offspring (Liu et al., 2007; Srám et al., 1999; Yokota et al., 2013). Therefore, reproductive and developmental toxicity has been recently raised as an important issue among the concerns about the

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potential adverse health effects following exposure to NPs (Campagnolo et al, 2012; El-Sayed et al, 2015; Hougaard et al., 2015; Kadar et al., 2013). However, only very little information is still available about them, especially in mammals (Ema et al., 2010).

FeNPs induced programmed cell death by generating oxidative stress in immune cells (Couto et al., 2014; Park et al., 2015) and altered immune homeostasis in body remaining in the body for a long time (Ban et al., 2013; Park et al., 2010). Additionally, occurrence of hyperresponsiveness disease in early-life following exposure to environmental risk factors increased rapidly with industrial development and has been considered as a major health problem worldwide (Caminati et al., 2015; Huang et al., 2015; Miller and Peden, 2014; Wegienka et al., 2015). In our previous study, we found that pulmonary FeNPs (0.5, 1, and 2 mg/kg) induces Th1-type immune response, stimulating function of antigen presenting cells on day 90 after a single intratracheal instillation (Park et al., 2015). In this study, we aimed to identify reproductive toxicity and developmental immunotoxicity following consecutive pulmonary exposure of FeNPs to the parent and their offspring mice. Soluble iron of 20 mg/kg dose is toxic for humans (Velez and Delaney, 2006). Therefore, we a single instilled intratracheally FeNPs (1, 2 and 4 mg/kg) to the parent mice and performed reproductive toxicity according to the modified Organization for Economic Co-operation and Development (OECD) TG 421. Additionally, we a single instilled intratracheally the same dose of FeNPs to their offspring mice (5 weeks-old) and observed the immunotoxic response on day 28.

2. Material and methods

2.1. Preparation of FeNPs

As reported previously (Park et al., 2014, 2015), FeNPs was prepared by the well-known reduction method, which was suggested by Kang et al. (1996). The synthesis process of FeNPs is follows; $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ were dissolved in HCl solution (0.04 M), and then Fe precursor solution was added to NaOH solution by drop-wise method (1.5 M). After the first drop of Fe solution, the color of mixture was gradually changed from brown to black. The mixture was left for 5 min after addition of NaOH solution. The precipitated FeNPs was separated by centrifugation (13,000 rpm), and washed 3 times with deionized water (DW). The resulting FeNPs was re-dispersed in DW, and then HCl solution (0.01 M) was added in FeNPs solution. The prepared was heated at boiling temperature and HCl solution (1 M) was added for 3 min. The solution was cooled down to room temperature (RT), and the rod-shaped FeNPs, known as FeOOH (iron oxyhydroxyde) NPs, was finally obtained (Fig. 1). Hydrodynamic diameter of FeNPs in phosphate-buffered saline (PBS), a vehicle used for dosing, and Gamble's solution, an artificial lung fluid, were 209.4 ± 98.0 and 45.1 ± 2.6 nm, respectively, and their surface charges were 11.9 ± 2.6 and −219.1 ± 14.0, respectively.

2.2. Animal care and FeNPs treatment

ICR mice (6-weeks) were obtained from Orient Bio Inc. (Gyeonggi-do, Korea) and acclimated for 1 week in our specific-pathogen-free facility (23 ± 3 °C, relative humidity of 50 ± 10%, 12 h light/dark cycle, and ventilation of 10–20 times/h). The parent mice (10 mice/dose/sex) and their offspring mice (5-weeks, female (21–24 g) and male (27–29 g), 12 mice/dose/sex) were a single exposed intratracheally to FeNPs (1, 2 and 4 mg/kg, Fig. 2), and the control group was instilled with a vehicle which was made by performing the same steps without the iron compounds. The experiment (IACUC No. 2014-0037) was assessed by the Institutional Animal Care and Committee (IACUC) of Ajou University (Suwon, Korea) and performed in accordance with the "Guide for the Care and Use of Laboratory Animals", an Institute for Laboratory Animal Research publication. Changes in body weight of parent and offspring mice following exposure to FeNPs were checked one time per week, and development of offspring mice following exposure to the parent mice was evaluated on 6–12 h, day 14 and day 21 after birth.

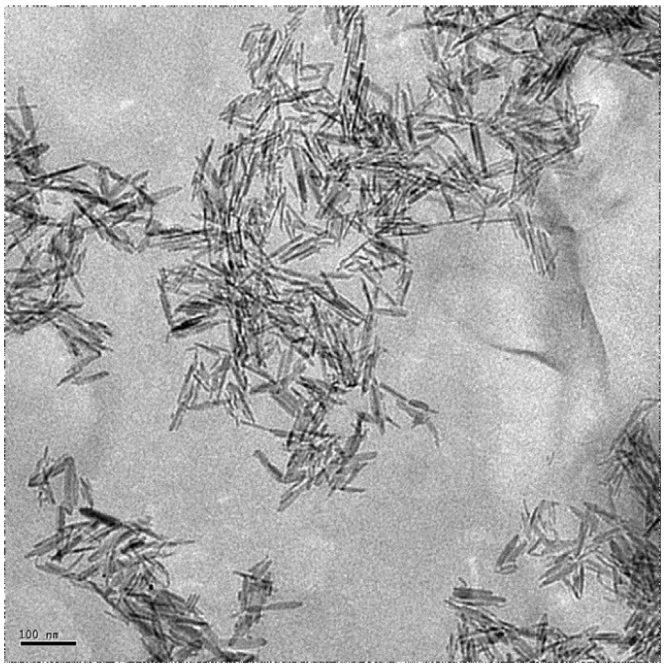

Fig. 1. TEM image of FeNPs.

2.3. Blood analysis

Blood was obtained via postcaval vein and a part of whole blood was centrifuged at 3000 rpm for 10 min to obtain serum for biochemical analysis. Hematological and biochemical analysis were performed using a blood autoanalyzer (HemaVet850, CDC Technologies, Inc., Dayton, Ohio, USA) and chemistry analyzer (BS-400, Mindray, Shenzhen, China), respectively, in Neodin Veterinary Science Institute (Seoul, Korea).

2.4. Histopathological analysis

The tissues were obtained from the parent (ovaries and testes, 5 samples/group) and offspring (lungs, 4 samples/group) mice under carbon dioxide gas anesthesia. The ovaries and lungs and the testes were fixed in 10% neutral buffered formalin and Bouin's solution, respectively. All the process for the evaluation of histopathological lesion was performed according to the standard operating procedures of the Korea Institute of Toxicology (Daejeon, Korea), a good laboratory practice institute in Korea.

2.5. Microarray and ingenuity pathway analysis

To investigate effects of the male parent mice to reproductive toxicity, the testes were obtained from the male parent mice of the control and the highest dose of group after mating, and microarray analysis (Macrogen Inc., Seoul, Korea) was performed using Illumina MouseRef-8 v2 Expression BeadChip (Illumina, Inc., San Diego, CA, USA) as previously described method (Park et al., 2014). Finally, ingenuity pathway analysis (IPA) was performed using microarray data (Macrogen Inc., Seoul, Korea).

2.6. Iron staining of tissue sections

The ovaries and the testes from the parent mice were embedded in

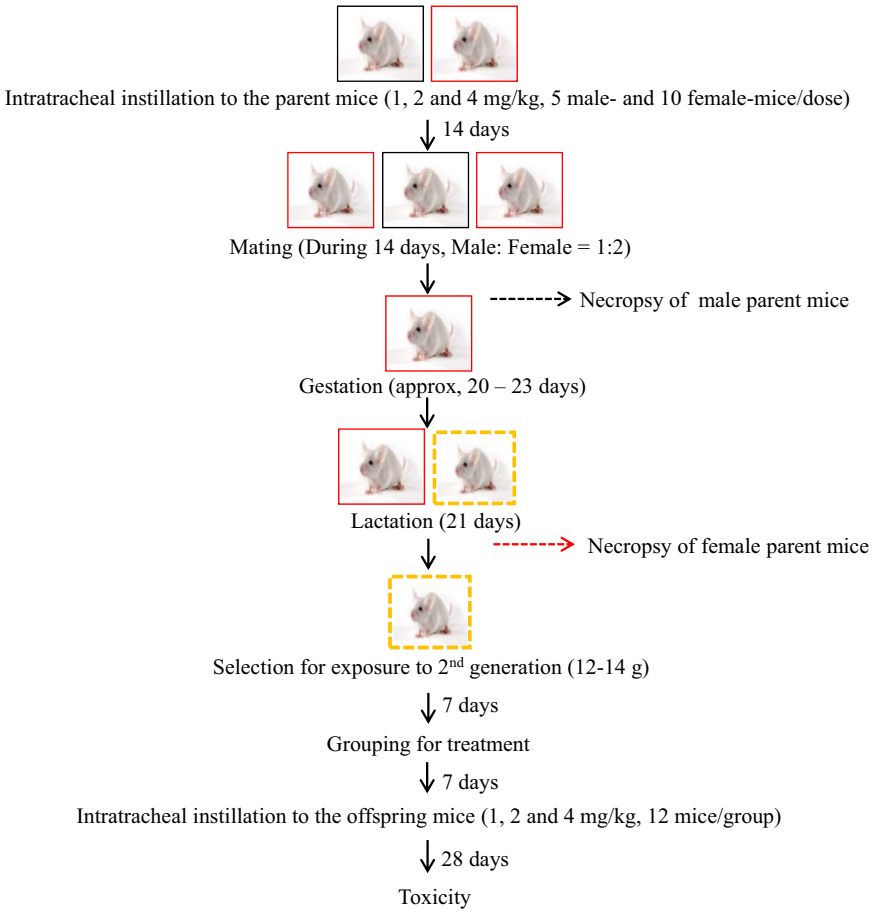

Fig. 2. An experimental design for this study.

paraffin, cut into 4-μm sections, and placed on slides. After deparaffinizing, the sections were covered with 10% ammonium sulfide ($(NH_4)_2S$, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at RT and incubated for 5 min in DW. Sections were stained with potassium ferricyanide hydrochloride ($K_3Fe(CN)_6$/HCl, Sigma-Aldrich) for 15 min at RT and then incubated in Kernechtrot solution (Sigma-Aldrich) for 5 min at RT. After washing with DW, sections were dehydrated by sequentially incubating them for 3 min each in 70%,

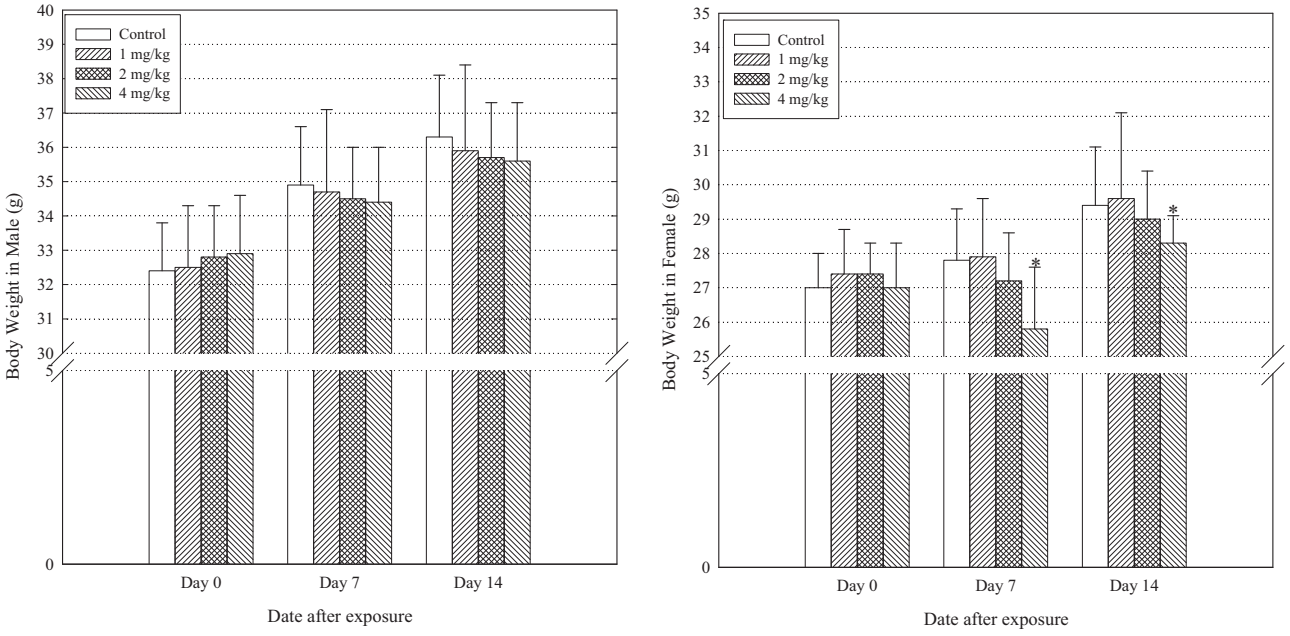

Fig. 3. Changes in body weight of parent mice following exposure to FeNPs. Body weight was measured weekly, and 2 female mice died on day 7 and 10 after instillation. Data are mean ± standard deviation (SD) of each group. Additionally, we calculated body weight gain using body weight of mice from treatment and necropsy times. Then, we conducted statistical analysis on body weight gain during experimental period. *$p < 0.05$.

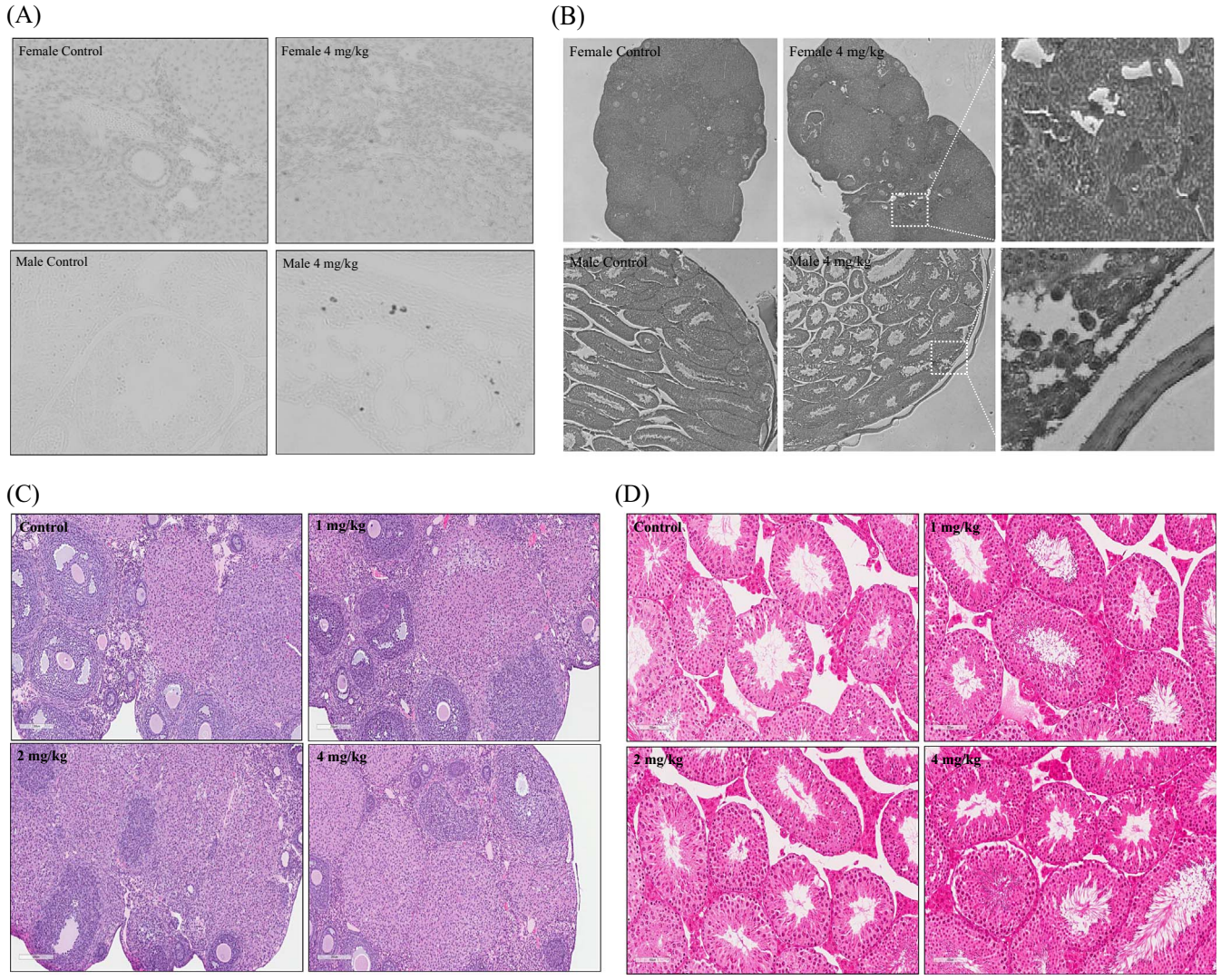

Fig. 4. Changes in the reproductive organs of parent mice. The ovaries and the testes from parent mice (5 mice/group) were stained and representative images were presented. (A) Increased iron distribution. Blue color indicates accumulation of iron. (B) Increased expression of MHC class II protein (C) Histopathological image of the ovary (D) Histopathological image of the testes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

80%, 96% and 100% ethanol, and xylene. Sections were mounted using a xylene-based mounting medium, and finally the slides were imaged under a microscope (LSM710, Carl Zeiss, Germany).

2.7. Immunohistochemistry

Prior to the staining process, the paraffin embedded-sections (the ovaries and testes) were deparaffinized in xylene and rehydrated through a sequential alcohol gradient. After washing with DW, the sections were incubated in tris-ethylene diamine tetraacetic acid (EDTA) buffer (10 mM Tris base, 1 mM EDTA, and 0.05% Tween-20, pH 9.0), heated in a microwave for antigen retrieval, and then incubated in 3% hydrogen peroxide for 10 min for inactivation of endogenous peroxidase. After washing with PBS, the sections were blocked with 3% bovine serum albumin in PBS for 1 h at RT and incubated with an MHC class II antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4 °C. Then, the sections were incubated with a horseradish peroxidase-conjugated antibody for 3 h at RT, counterstained with Mayer's hematoxylin (Dako, Carpinteria, CA, USA) and sequentially incubated in ethanol gradients and xylene. The coverslips were mounted using permount, and the slides were imaged under a microscope (LSM710, Carl Zeiss).

2.8. Total cell counts and cell cycle analysis

Bronchial alveolar lavage (BAL) fluids were obtained by cannulating the trachea with a blunted 24-gauge needle and lavaging the lungs twice with cold sterile PBS (1 ml) on day 28 after a single intratracheal instillation to the offspring mice. After centrifuging at 1500 rpm for 5 min, the pellets were used for total cell counts and cell cycle analysis, and the supernatants were used for the cytokine assay. The total cell number in the BAL fluid was countered under a microscope (LSM710, Carl Zeiss). Additionally, BAL cells were fixed with 70% ethanol, washed with fluorescence-activated cell sorting (FACS) buffer, treated with RNase A (200 μg/ml, Sigma-Aldrich) for 30 min, and then stained with propidium iodide (20 μg/ml, Sigma-Aldrich) for DNA labeling. Cell cycle was analyzed by measuring the DNA content using the FACSCalibur system and CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

2.9. Cytokine assays

Concentration of pro-inflammatory cytokines (IL-1β, tumor necrosis factor (TNF)-α, IL-6), Th1-type cytokines (IL-12/IL-23) and Th2-type cytokines (IL-4 and 10) was measured using commercially available enzyme-linked immunosorbent assay kits (eBioscience, San

Table 1
Changes in gene profile in testes of male parent mice exposed to FeNPs. The testes of male parent mice were collected from 6 mice per group for microarray analysis and those from two mice were pooled equivalently to make one sample for analysis (n=3 samples/group). List shows genes, which were altered more than 1.7-fold with statistical significance ($p < 0.05$). (A) Up-regulated and (B) down-regulated gene list.

Table 1(A)

RefSeq_NM	DEFINITION	Fold
NM_028243.2	prolylcarboxypeptidase (angiotensinase C)	3.18
NM_029150.1	spermatogenesis associated 16, transcript variant 2.	2.79
NM_027436.2	mitochondrial intermediate peptidase	2.74
NM_021285.1	myosin, light polypeptide 1	2.59
NM_007817.2	cytochrome P450, family 2, subfamily f, polypeptide 2	2.47
NM_207105.1	histocompatibility 2, class II antigen A, beta 1	2.46
NM_026302.3	dynactin 4	2.32
NM_026218.2	FGFR1 oncogene partner 2	2.22
NM_008318.1	integrin binding sialoprotein	2.18
NM_009606.2	actin, alpha 1, skeletal muscle	2.11
NM_024236.1	quinoid dihydropteridine reductase	2.09
NM_011305.3	retinoid X receptor alpha	2.07
XM_918601.3	PREDICTED: similar to MHC class II antigen beta chain	2.06
NM_028963.2	RIKEN cDNA 4933427D14 gene	2.04
NM_152817.3	tetratricopeptide repeat domain 27	2.04
NM_013645.3	parvalbumin	2.02
NM_174885.3	a disintegrin and metallopeptidase domain 6	1.98
NM_025654.2	RAD52 motif 1	1.94
NM_153409.3	cysteine-serine-rich nuclear protein 3	1.79
NM_029320.2	progesterone immunomodulatory binding factor 1, transcript variant 1	1.78
NM_021388.3	exostoses (multiple)-like 2	1.70

Table 1(B)

ACCESSION	DEFINITION	Fold
NM_178914.3	spermatogenesis associated 7	−3.17
NM_178914.3	spermatogenesis associated 7	−3.03
NM_178213.3	histone cluster 2, H2ab	−2.30
NM_007402.2	a disintegrin and metallopeptidase domain 7	−2.24
NM_026968.2	mannosidase, beta A, lysosomal-like	−2.18
NM_012031.1	sperm associated antigen 1	−2.14
NM_001014397.1	predicted gene, OTTMUSG00000010673	−2.10
NM_033268.3	actinin alpha 2	−2.03
NM_013459.1	complement factor D (adipsin)	−1.98
NM_008522.3	lactotransferrin	−1.91
NM_011755.2	zinc finger protein 35	−1.87
NM_008218.2	hemoglobin alpha, adult chain 1	−1.87
NM_023456.2	neuropeptide Y	−1.84
NM_194055.1	RNA binding motif protein 35 A	−1.83
NM_027347.2	mediator complex subunit 23	−1.83
NM_025387.2	transmembrane protein 14 C	−1.83
NM_007791.4	cysteine and glycine-rich protein 1	−1.81
NM_026976.2	Fas apoptotic inhibitory molecule 3	−1.80
NM_177612.1	catenin (cadherin associated protein), alpha 3	−1.78
NM_026496.3	grainyhead-like 2 (Drosophila)	−1.76
NM_025920.3	THAP domain containing 4	−1.72

Table 2
A summary of reproductive toxicity of parent mice exposed to FeNPs.

	Pregnant female	Female pups	Male pups	Total pups	Average pups	Sex ratio
Control	10	67	62	129	12.9	0.93
1 mg/kg	10	59	62	121	12.1	1.05
2 mg/kg	10	57	65	122	12.2	1.14
4 mg/kg	8	44	57	101	12.6	1.30

Diego, CA, USA) according to the manufacturer's instructions. Finally, the reactions were stopped by adding 1 M H_3PO_4, and the absorbance at 450 nm was measured using an ELISA reader (Molecular Devices). The concentration of each cytokine was calculated from the linear portion of the standard curve that was generated under the same

Table 3
Hematological changes in blood of offspring mice exposed to FeNPs. Blood was collected on day 28 after a single intratracheal instillation. Asterisks (*) indicate statistical significance of the treated-groups compared to the control (*$p < 0.05$, **$p < 0.01$). WBC, white blood cells; LY, lymphocytes; MO, monocytes; NE, neutrophils; EO, eosinophils; BA, basophils; RBC, red blood cells; MCV, mean corpuscular volume; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Hgb, hemoglobin; RDW, red blood cell distribution width; PLT, platelet; MPV, mean platelet volume.

Table 3(A)

Male	WBC	LY	MO	NE	EO	BA	RBC	MCV	HCT	MCH	MCHC	Hgb	RDW	PLT	MPV
Unit	K/uL	%	%	%	%	%	M/mm3	fl	%	pg	g/dL	g/dL	%	K/uL	fL
Control (n=12)	2.5 ± 0.7	85.8 ± 3.2	3.5 ± 1.4	6.5 ± 1.7	3.8 ± 1.2	0.5 ± 0.3	8.0 ± 0.4	50.1 ± 2.8	41.9 ± 2.6	16.3 ± 0.3	31.7 ± 0.6	13.3 ± 0.7	17.9 ± 0.7	1040.5 ± 87.5	2.5 ± 0.7
1 mg/kg (n=12)	2.8 ± 0.6	85.1 ± 3.7	2.7 ± 0.9	6.7 ± 2.6	4.6 ± 1.3	0.9 ± 0.3	8.3 ± 0.8	49.6 ± 2.8	41.2 ± 6.5	17.0 ± 1.2	34.3 ± 3.3	14.0 ± 1.3	18.1 ± 0.5	954.6 ± 108.3	2.8 ± 0.6
2 mg/kg (n=12)	2.8 ± 0.5	83.8 ± 2.9	2.3 ± 1.5	8.1 ± 3.9	5.1 ± 0.5	0.7 ± 0.5	7.5 ± 0.9	49.6 ± 1.5	37.0 ± 4.3	18.1 ± 1.6	36.4 ± 3.2*	13.4 ± 1.3	18.0 ± 0.3	870.9 ± 169.4	2.8 ± 0.5
4 mg/kg (n=9)	2.3 ± 0.4	79.2 ± 2.9*	3.2 ± 0.7	10.8 ± 2.2*	5.4 ± 2.2	1.4 ± 0.2**	7.9 ± 0.4	49.7 ± 2.2	39.2 ± 1.2	17.2 ± 0.9	34.6 ± 2.1*	13.6 ± 0.8	17.6 ± 0.6	944.3 ± 27.2	2.3 ± 0.4

Table 3(B)

Female	WBC	LY	MO	NE	EO	BA	RBC	MCV	HCT	MCH	MCHC	Hgb	RDW	PLT	MPV
Unit	K/uL	%	%	%	%	%	M/mm3	fl	%	pg	g/dL	g/dL	%	K/uL	fL
Control (n=12)	2.1 ± 1.1	87.9 ± 5.2	3.9 ± 0.9	6.4 ± 6.0	2.3 ± 1.3	0.4 ± 0.3	8.1 ± 0.3	50.6 ± 1.4	40.9 ± 1.8	16.9 ± 0.6	33.5 ± 1.4	13.7 ± 0.7	17.8 ± 0.4	936.7 ± 158.7	7.6 ± 0.6
1 mg/kg (n=12)	1.9 ± 1.1	83.3 ± 6.3	3.7 ± 0.8	10.3 ± 6.4	2.6 ± .8	0.8 ± 0.3	8.5 ± 0.9	50.3 ± 2.6	42.6 ± 5.9	16.8 ± 0.3	33.5 ± 1.4	14.2 ± 1.5	17.7 ± 0.7	701.6 ± 137.3	8.7 ± 1.5
2 mg/kg (n=10)	1.8 ± 1.3	79.6 ± 4.8	2.7 ± 1.1	12.1 ± 7.3	3.1 ± 3.1	1.1 ± 0.3*	7.5 ± 0.4*	49.9 ± 1.2	37.6 ± 2.3	17.2 ± 1.0	34.3 ± 1.8	12.9 ± 0.6	17.7 ± 0.4	694.6 ± 95.6*	7.8 ± 0.5
4 mg/kg (n=8)	0.7 ± 0.2**	66.0 ± 12.7*	2.4 ± 0.5*	25.4 ± 3.8**	4.5 ± 4.5	1.8 ± 0.5**	5.7 ± 1.5**	50.3 ± 2.2	29.1 ± 8.7*	19.8 ± 6.7	43.7 ± 9.5	11.9 ± 1.4	17.0 ± 0.3	493.6 ± 60.7**	7.6 ± 0.8

Table 4
Biochemical changes in blood of offspring mice exposed to FeNPs. Asterisks (*) indicate statistical significance of the treated-groups compared to the control (*$p < 0.05$, **$p < 0.01$). TP; Total protein, TB; Total bilirubin, Cr; Creatinine, AST; Aspartate aminotransferase, ALT; Alanine aminotransferase, ALP; Alkaline phosphatase, γ-GTP; Gamma-glutamyl transferase, Na; Sodium, K; Potassium, Cl; Chloride.

Table 4(A)

Male	TP	Albumin	TB	Glucose	BUN	Cr	AST	ALT	ALP	γ-GTP	Amylase	Globulin	Na	K	Cl
Unit	g/dL	g/dL	mg/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	U/L	U/L	g/dL	mmol/L	mmol/L	mmol/L
Control (n=12)	4.5 ± 0.3	3.3 ± 0.2	0.1 ± 0.0	362.0 ± 51.6	19.9 ± 0.8	0.3 ± 0.0	38.0 ± 3.2	26.0 ± 1.6	69.5 ± 5.0	0.0 ± 0.0	2804.9 ± 257.4	1.2 ± 0.1	144.9 ± 3.7	5.9 ± 0.2	106.5 ± 2.7
1 mg/kg (n=12)	4.3 ± 0.3	3.1 ± 0.1	0.1 ± 0.0	344.3 ± 28.8	19.6 ± 1.6	0.2 ± 0.0	39.8 ± 3.5	29.5 ± 2.5	68.5 ± 6.2	0.0 ± 0.0	3029.1 ± 307.4	1.2 ± 0.2	143.5 ± 2.2	6.0 ± 0.4	105.7 ± 1.4
2 mg/kg (n=12)	4.6 ± 0.1	3.3 ± 0.0	0.1 ± 0.0	305.3 ± 61.8	21.2 ± 2.0	0.3 ± 0.0	43.8 ± 6.8	31.0 ± 4.8	89.0 ± 11.1	0.0 ± 0.0	2739.9 ± 71.7	1.3 ± 0.1	144.8 ± 1.0	5.4 ± 0.2*	107.0 ± 1.7
4 mg/kg (n=9)	5.2 ± 0.3*	3.7 ± 0.2	0.1 ± 0.0	297.0 ± 30.4*	18.1 ± 1.5	0.3 ± 0.0	110.8 ± 56.4**	47.5 ± 3.8**	51.5 ± 5.7*	0.0 ± 0.0	2602.5 ± 159.6	1.5 ± 0.1*	145.0 ± 0.3	4.4 ± 0.0**	113.9 ± 15.6

Table 4(B)

Female	TP	Albumin	TB	Glucose	BUN	Cr	AST	ALT	ALP	γ-GTP	Amylase	Globulin	Na	K	Cl
Unit	g/dL	g/dL	mg/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	U/L	U/L	g/dL	mmol/L	mmol/L	mmol/L
Control (n=12)	4.6 ± 0.2	3.4 ± 0.1	0.1 ± 0.0	327.4 ± 44.1	20.4 ± 1.6	0.5 ± 0.0	79.3 ± 14.2	29.8 ± 3.9	81.0 ± 11.6	0.0 ± 0.0	3287.4 ± 175.5	1.2 ± 0.1	152.7 ± 3.7	6.3 ± 0.7	114.9 ± 3.0
1 mg/kg (n=12)	4.0 ± 0.2	3.1 ± 0.1	0.1 ± 0.0	383.0 ± 70.1	18.2 ± 3.5	0.4 ± 0.0	57.2 ± 8.6*	36.6 ± 6.5	91.0 ± 8.1	0.0 ± 0.0	2815.6 ± 262.0	0.9 ± 0.2	154.4 ± 4.1	6.2 ± 0.7	116.0 ± 2.1
2 mg/kg (n=10)	4.4 ± 0.2	3.3 ± 0.2	0.1 ± 0.0	380.6 ± 49.9	18.8 ± 3.5	0.5 ± 0.0	50.6 ± 9.1**	23.4 ± 3.3	79.3 ± 15.7	0.0 ± 0.0	2975.8 ± 306.7	1.2 ± 0.1	149.6 ± 1.8	5.3 ± 0.2*	109.5 ± 1.4*
4 mg/kg(n=8)	4.5 ± 0.1	3.3 ± 0.1	0.1 ± 0.0	402.3 ± 53.4*	22.3 ± 3.0	0.4 ± 0.1	41.4 ± 8.8**	23.0 ± 2.4*	68.2 ± 9.5	0.0 ± 0.0	2547.9 ± 158.8*	1.1 ± 0.1	146.7 ± 2.3*	4.8 ± 0.2**	107.8 ± 1.7**

condition.

2.10. Immunophenotypic analysis

The spleen was collected from offspring mice on day 28 after instillation, and splenocytes were isolated in RPMI media containing fetal bovine serum (2%). After removing red blood cells (RBC) with RBC lysis solution (BD Biosciences), splenocytes were filtered using a 70 μm-pore size nylonmesh (SPL Life Sciences, Gyeonggi-do, Korea), and then were resuspended in FACS buffer. Following, splenocytes and BAL cells (4 mice/group) were blocked with cluster of differentiation (CD)16/CD32 antibody (eBiosciences) to reduce nonspecific binding, then incubated with the fluorescence dye labeled antibodies: phycoerythrin (PE)-conjugated anti-CD11b, fluorescein isothiocyanate (FITC)-conjugated anti-CD11c, allophycocyanin (APC)-conjugated anti-MHC Class II (I-A/I-E), APC- conjugated anti-CD40, FITC- conjugated anti-CD3, PE- conjugated anti-CD19, APC-conjugated anti-DX5, PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and FITC-conjugated anti-CD80 (B7-1) (eBioscience); APC-conjugated anti-CD86 (B7-2, BioLegend, Inc. San Diego, CA, USA) for 30 min at 4 °C according to the manufacturer's instructions. Cells were washed twice with FACS buffer and analyzed on a FACSCalibur flow cytometer with CellQuest software.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses of the treated-groups compared to the control were performed using Student's *t*-test (Graphpad Software, San Diego, CA, USA) and one way ANOVA test followed by Tukey's post hoc pairwise comparison. Asterisks (*) indicate statistically significant differences.

3. Results

3.1. Effects on the parent mice

During the pre-mating period, 2 female mice died at a 4 mg/kg dose (day 7 and 10 after instillation), and the body weight gain decreased in a dose-dependent manner in FeNPs treated-mice of both sexes compared to the control (Fig. 3). The average body weight gain was 2.4, 2.2, 1.6 and 1.3 g for the control and the 1, 2 and 4 mg/kg-treated female parent mice, respectively, and that was 3.9, 3.4, 2.9 and 2.7 for the control and the 1, 2 and 4 mg/kg-treated male parent mice, respectively. Additionally, we found that iron accumulation (Fig. 4A) and expression of MHC class II protein (Fig. 4B), a representative antigen-presenting related-molecule, are enhanced in the ovaries and testes following exposure to FeNPs (4 mg/kg). Moreover, the expression of MHC class II-related genes and cytochrome P450, a hemoprotein enzyme, significantly increased in the testes of mice exposed to 4 mg/kg dose of FeNPs (Table 1). On the other hand, we could not find remarkable histopathological changes following exposure to FeNPs in the ovaries (Fig. 4C) and the testes (Fig. 4D).

3.2. Effects on offspring mice at birth

The gestation rate and the birth number per mice did not show significant difference between groups following exposure to FeNPs (Table 2). Namely, all the live female parent mice was pregnant, and the birth number per mouse was 12.9 ± 3.3, 12.1 ± 3.4, 12.2 ± 2.0 and 12.6 ± 3.5 for the control and 1, 2 and 4 mg/kg dose group, respectively. Each one mouse died within 12 h after birth at 1 and 2 mg/kg dose. Meanwhile, noteworthy, the total sex ratio (male/female) increased following exposure to FeNPs (Table 2).

3.3. Effects on development of the offspring mice

There were no remarkable differences in body weight of offspring mice at the birth between groups (Fig. S1). Meanwhile, as compared to

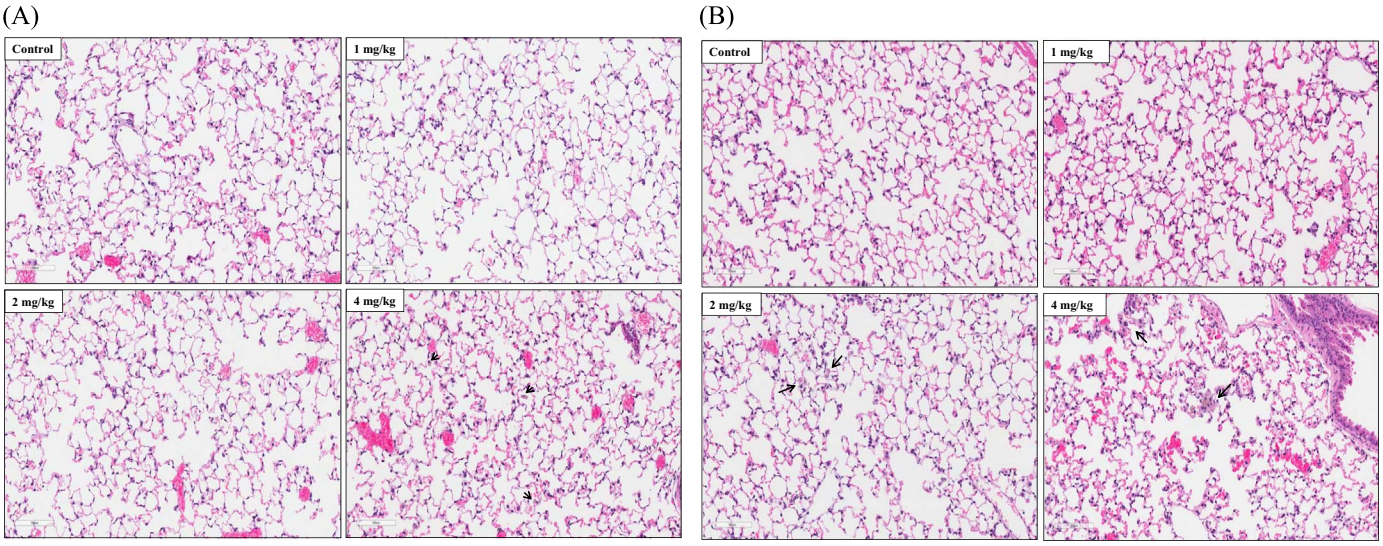

Fig. 5. Histopathological lesion in the lungs of offspring mice exposed to FeNPs. The lung tissues of offspring mice (4 mice/group) were fixed in 10% neutral-buffered formalin and arrows indicate pulmonary persistence of FeNPs. Representative images by dose are shown. (A) Male (B) Female.

the control, development of offspring mice (body weight gain) rather tended to be enhanced in the FeNPs treated-group. Namely, the body weight of offspring mice was 6.9 ± 0.9, 6.9 ± 1.3, 7.9 ± 1.2, and 7.5 ± 0.9 g for the control and 1, 2, and 4 mg/kg dose groups, respectively, on day 14 after birth, and that was 12.3 ± 1.3, 13.3 ± 1.7, 14.0 ± 1.6, and 13.1 ± 1.8 g for the same groups, respectively, on day 21 after birth. Here, we speculated that this might be attributed to the increased number of male mice in the treated groups.

3.4. General toxicity in the offspring mice following exposure to FeNPs

Following, we aimed to evaluate the toxic response which induced in offspring mice, especially in young offspring mice, when FeNPs were exposed consecutively to two generation. For this, offspring mice (5-weeks, 12 mice/dose/sex) was a single instilled intratracheally with FeNPs of the same dose with that in the parent mice, and then general toxicity- and immunotoxicity-related endpoints were measured on day 28. During the observation period, 2 female mice died at 2 mg/kg dose, and 4 female and 3 male mice died at 4 mg/kg dose. The body weight increased in a similar pattern in offspring mice of both sexes after exposure, and there were no significant differences following exposure to FeNPs (Fig. S2). Meanwhile, compared to the control, the portion of neutrophils, eosinophils, and basophils was clearly enhanced in the blood of the male mice treated at 4 mg/kg dose (Table 3A). Similarly, as compared to the control, the decreased number of white blood cells, the reduced portion of lymphocytes and macrophages and the increased portion of neutrophils, eosinophils and basophils were significantly observed in the blood of the female offspring mice exposed to the highest dose (4 mg/kg). Additionally, remarkable reduction in the red blood cell count, hematocrit, hemoglobin level, and platelet count was observed in the female offspring mice exposed to the highest dose (4 mg/kg). Furthermore, when treated at 4 mg/kg dose, total protein, AST, ALT and globulin levels significantly increased in the blood of the male offspring mice compared to the control, whereas glucose, ALP and potassium levels clearly decreased (Table 4A). At the same dose, levels of AST, ALT, ALP, amylase, sodium, potassium and chloride remarkably decreased in the blood of the female offspring mice, whereas glucose level was elevated (Table 4B).

3.5. Immunotoxic response in the offspring mice following exposure to FeNPs

In the lung, we found that pigmented macrophages (Fig. 5) and total number of BAL cells (Fig. 6A) are dose-dependently elevated in offspring mice of both sexes exposed to FeNPs. Meanwhile, interestingly, apoptotic death of BAL cells increased only in lung of male offspring mice (Fig. 6B). More interestingly, levels of IL-1β, IL-6, IL-12, IL-4 and IL-10 were dose-dependently enhanced in BAL fluid of the female offspring mice, whereas only the IL-12 level was dose-dependently elevated in that of male offspring mice (Fig. 6C). Following, considering that nanoparticles can influence the function of antigen-presenting cells (APCs, Jiménez-Periáñez et al., 2013; Koike et al., 2008; Tkach et al., 2011), we investigated the effect of FeNPs on the function of APCs using BAL cells and splenocytes. As results, FeNPs dose-dependently augmented co-expression of CD80 and CD86 proteins on BAL cells of the female offspring mice, but significant changes were not observed in the male offspring mice (Fig. 6D). In addition, as compared to the control, the proportion of T cells was dominant in male offspring mice exposed to the highest dose, and cytotoxic and helper T cells were dominant in the male and female offspring mice, respectively (Fig. 7A). Moreover, at 4 mg/kg dose, co-expression of CD80 and CD86 proteins was attenuated on splenocytes of the female offspring mice compared to the control (Fig. 7B), and expression of the MHC class II remarkably reduced on splenocytes from mice of both sexes (Fig. 7B). However, the maturation of dendritic cells, a representative antigen-presenting cell, was not influenced in the offspring mice of both sexes following exposure to FeNPs (Fig. 7C).

4. Discussion

NPs have been widely applied in a variety of fields including advanced materials development, electronics, optoelectronics, biomedicine for diagnosis and therapy, pharmaceuticals, cosmetics, and energy, as well as catalytic and environmental detection and monitoring. Therefore, the frequency and amounts, which humans can be exposed to them during production, use and disposal process, is rapidly increasing. However, information about their potential toxicity, especially for high-risk people including pregnant women and infants, is still insufficient (Ema et al., 2010).

The biodistribution and bioaccumulation levels of NPs can be controlled by their unique physicochemical properties, including size, shape, surface charge, solubility, and functional groups (Almeida et al., 2011; De Jong et al., 2008; Lee et al., 2013; Yang et al., 2015), and previous reports show that NPs can accumulate in the reproductive organs. Additionally, accumulated NPs can affect the reproductive organs of parents, raising the potential of adverse health effects in the next generation. For example, Di Bona et al. (2014, 2015)

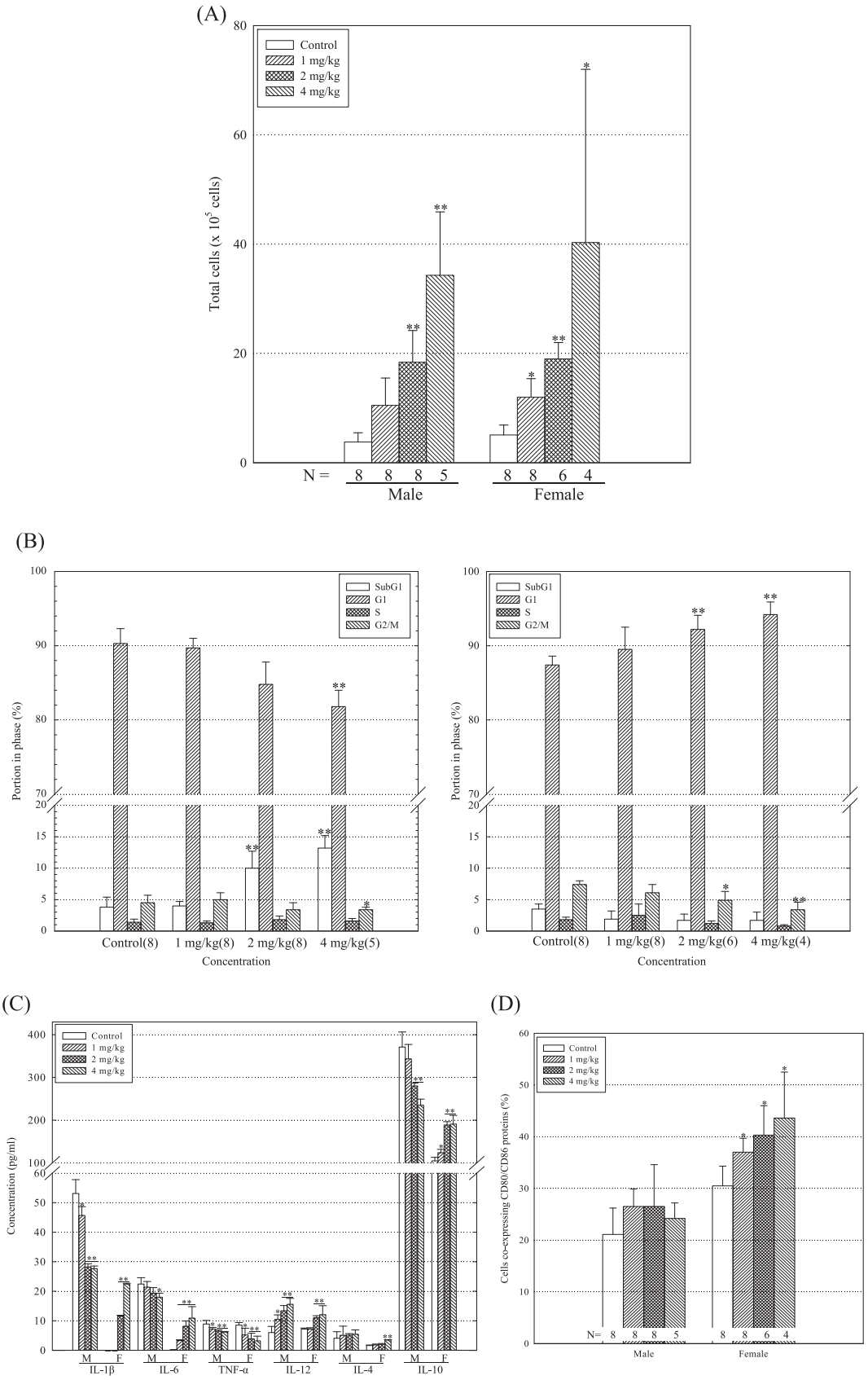

Fig. 6. Changes in the lung of offspring mice exposed to FeNPs. BAL fluids were harvested on day 28 after a single intratracheal instillation and data are mean ± SD of each group. *$p <$ 0.05, **$p < 0.01$. (A) Number of total cells. N=sample number (B) Changes in cell cycle of BAL cells. 5000 cells per sample were counted and sample number of each group is shown in parentheses. (C) Cytokine level in the lung. The values were calculated using a standard curve, which was constructed under the same conditions. (D) Co-expression of CD80 and CD86 proteins on BAL cells. A total of 5000 cells per sample were counted.

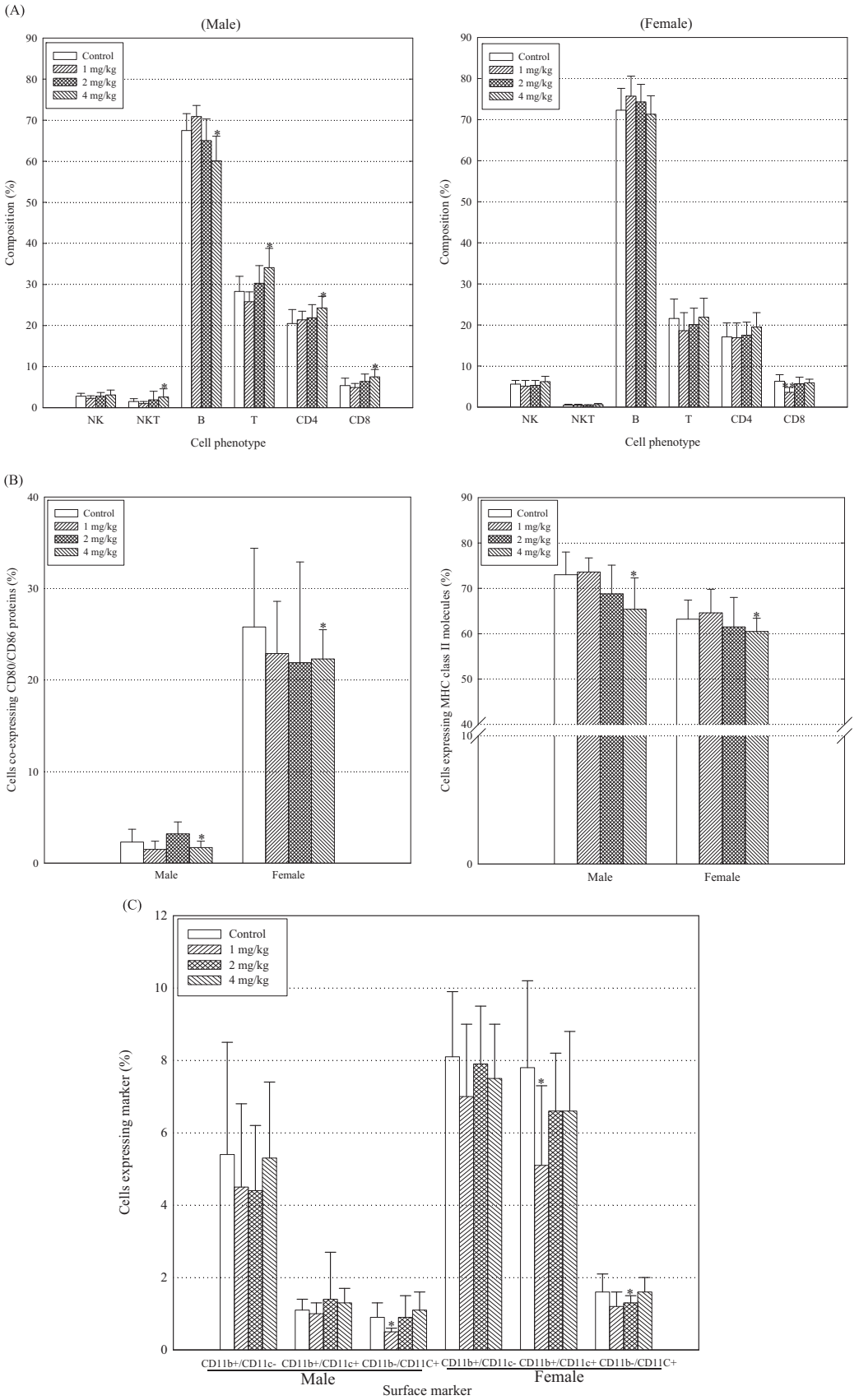

Fig. 7. Systemic immunotoxic effects following exposure to FeNPs. Spleens were collected from all mice alive during necropsy (control and 1 mg/kg, n=12 mice/group; 2 mg/kg, male, n=12, female, n=10 mice; 4 mg/kg, male, n=10, female, n=8). A total of 10,000 cells per sample were counted. $^{*}p < 0.05$, $^{**}p < 0.01$. (A) Composition of lymphocytes in splenocytes (B) Effect of expression of antigen presenting related-proteins (C) Effects on maturation of dendritic cells (DCs). $CD11b^{-}/CD11c^{+}$, regulatory DCs, $CD11b^{+}/CD11c^{+}$, immature DCs; $CD11b^{-}/CD11c^{+}$, mature DCs.

investigated effect of surface charge on developmental and reproductive toxicity of FeNPs using pregnant mice, and they reported that FeNPs which have positive charge show stronger toxic effects compared to them which have negative charge. Silver NPs also persisted in the brain and the testes for a long time due to their low clearance rate (Lee et al., 2013; van der Zande et al., 2012). Additionally, oral exposure to titanium dioxide (TiO_2) NPs (1 and 2 mg/kg) for 5 days resulted in accumulation of NPs in the spleen and the ovaries, and testosterone level increased and decreased in the highest dose of males and female offspring mice, respectively (Tassinari et al., 2014), and repeated intratracheal instillation of carbon black (14 and 56 nm, 0.1 mg/mouse, 10 times every week) elevated the serum testosterone levels and induced partial vacuolation of the seminiferous tubules in mice (Yoshida et al., 2009). Similarly, repeated injection of TiO_2 NPs (500 mg/kg, every other day, 5 times) significantly reduced the sperm density and motility, increased sperm abnormality and germ cell apoptosis, although no obvious pathological changes were observed in the testes (Guo et al., 2009). Additionally, FeNPs of 50 and 78 nm, but not 15 nm, resulted in a leaky epithelium and enhanced cell death, ROS generation, and disruption of junctional integrity in human placental cells (Faust et al., 2014), and maternal exposure to TiO2 NPs and carbon black led to adverse effects on spermatogenesis in the offspring (Ema et al., 2010). TiO_2 NPs were also transferred from pregnant mice to their offspring and affected the brain development of the offspring (Shimizu et al., 2009; Takeda et al., 2009). Furthermore, FeNPs, but not TiO_2 NPs, was clastogenic (Bhattacharya et al., 2009) and caused serious disruption of development, consisting of 30% mortality among spermatozoa with subsequent 20% decline in fertilization success and delay in development. In this study, elevated iron accumulation was observed in the ovaries and the testes of mice exposed to FeNPs (4 mg/kg), and expression of the MHC class II protein and upregulation of cytochrome P450 and MHC class II-related genes were enhanced in the same mice. MHC class II molecules are normally found only on APCs such as macrophages, B cells and dendritic cells (DCs), and extracellular proteins are endocytosed by phagocytic cells, digested in lysosomes, and the resulting peptides are presented on the cell surface with MHC class II molecules. In our previous studies, FeNPs induced autophagic cell death in macrophages, and some organelles existed in autophagolysosome-like vacuoles (Park et al., 2014). Additionally, cytochrome P450 belongs to the superfamily of proteins containing a heme cofactor. Herein, we can guess that FeNPs themselves or FeNPs-taken up macrophages were transferred into the ovaries and testes of mice exposed to FeNPs. Furthermore, we found that the expression of endocrine system disorders-, gastrointestinal disease-, inflammatory disease-, metabolic disease- and immunological disease-related genes were the most altered in the testes of the male parent mice exposed to FeNPs (4 mg/kg dose, Fig. S3). These genes are also involved in the development and function of the hematological system and hematopoiesis. Noteworthy, the ratio of male to female increased in FeNPs-treated groups, although information on statistical significance was not obtained due to our mistake in the experimental process. In a previous report, the sex ratio of storks was altered based on the degree of environmental degradation of cadmium and lead (Kamiński et al., 2015). Additionally, in the current study, female more died than male mice following exposure to FeNPs. Herein, we suggest that further studies are necessary to elucidate the detailed mechanism underlying the regulation of sex ratio by FeNPs (Di Bona et al., 2014, 2015).

Generally, foreign bodies can initiate an innate immune response in the body by stimulating phagocytic cells such as macrophage and neutrophils until day 3–7 after their entry. In addition, adaptive immune responses are induced by the stimulation of APCs since day 7 after entry, and the expression of CD80, CD86, MHC class II and CD40 proteins is augmented during this process. Recently, some researchers suggested that NPs can influence immune regulation by altering the function of APCs, especially DCs (Jiménez-Periáñez et al., 2013; Koike et al., 2008; Tkach et al., 2011). Additionally, it is known that newborns are more susceptible to the attack by foreign bodies because their immune systems are immature. Furthermore, accumulating evidence shows that maternal exposure to hazardous materials influences the development of the immune system of newborns. For example, the exposure of mothers to carbon black during early gestation partially suppressed the development of the immune system of offspring mice, and the decreased splenic T cells in the carbon black-treated group was recovered at 14 days after birth (Shimizu et al., 2014). Additionally, pregnant mice exposed to TiO_2 NPs secreted higher serum levels of cytokines than non-pregnant mice, and their offspring mice showed increased airway hyperresponsiveness, increased percentage of eosinophils, and pulmonary inflammation (Fedulov et al., 2008). In our previous study, FeNPs did not induce death in any the treated-male mice up to day 90 after a single intratracheal instillation (0.5, 1 and 2 mg/kg, Park et al., 2015), and apoptotic death of BAL cells and Th1-polarized inflammatory response were observed in the lungs of FeNPs-treated mice. Moreover, the secretion of chemokines, including granulocyte macrophage colony-stimulating factor, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1, was elevated in BAL fluid, and the expression of antigen presentation-related proteins, including CD80, CD86, and MHC class II, was enhanced on APCs in the lung. Meanwhile, in the current study, FeNPs (2 and 4 mg/kg) induced death in the female parent mice and the offspring mice of both sexes, and the mortality was clearly higher in offspring mice than the parent mice and in female mice than male mice. The hematological and biochemical alterations were also more remarkable in the blood of female offspring mice than in that of male offspring mice. More interestingly, apoptotic death of BAL cells following exposure to FeNPs was observed only in male offspring mice accompanying relative increase of cytotoxic T cells, and co-expression of CD80 and CD86 proteins on BAL cells was enhanced only in female offspring mice with a relative increase of helper T cells. Additionally, on day 28 after a single instillation, FeNPs (4 mg/kg) systemically stimulated innate immune responses and attenuated the function of APCs, especially in the female groups, although they did not influence maturation of splenic DCs. Considering that FeNPs can generate free radicals, which are immunologically active and biologically toxic (Couto et al., 2014; Park et al., 2014), that lymphocytes can alter the antioxidant level when exposed to NPs, and that FeNPs require special reducing conditions before pronounced radical generation (Bhattacharya et al., 2009), we suggest that further study are necessary to identify causes of the different immune responses induced in offspring mice of both sexes.

In conclusion, our results suggest that the no-observed-adverse-effect level (NOAEL) for reproductive and developmental toxicity of FeNPs may be lower than 2 mg/kg, and that female mice are more sensitive than male mice for exposure to FeNPs. This study also demonstrates that more careful assessment of FeNPs is required before their application.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2016.08.025.

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