Research Article

Journal of Applied Toxicology

Received: 23 November 2014, Revised: 14 December 2014, Accepted: 23 December 2014 Published online in Wiley Online Library: 6 March 2015

(wileyonlinelibrary.com) DOI 10.1002/jat.3125

Comparative toxicity of silicon dioxide, silver and iron oxide nanoparticles after repeated oral administration to rats

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ABSTRACT: Although silicon dioxide (SiO_2), silver (Ag) and iron oxide (Fe_2O_3) nanoparticles are widely used in diverse applications from food to biomedicine, *in vivo* toxicities of these nanoparticles exposed via the oral route remain highly controversial. To examine the systemic toxicity of these nanoparticles, well-dispersed nanoparticles were orally administered to Sprague–Dawley rats daily over a 13-week period. Based on the results of an acute toxicity and a 14-day repeated toxicity study, 975.9, 1030.5 and 1000 mg kg^{-1} were selected as the highest dose of the SiO_2, Ag and Fe_2O_3 nanoparticles, respectively, for the 13-week repeated oral toxicity study. The SiO_2 and Fe_2O_3 nanoparticles did not induce dose-related changes in a number of parameters associated with the systemic toxicity up to 975.9 and 1000 mg kg^{-1}, respectively, whereas the Ag nanoparticles resulted in increases in serum alkaline phosphatase and calcium as well as lymphocyte infiltration in liver and kidney, raising the possibility of liver and kidney toxicity induced by the Ag nanoparticles. Compared with the SiO_2 and Fe_2O_3 nanoparticles showing no systemic distribution in all tissues tested, the Ag concentration in sampled blood and organs in the Ag nanoparticle-treated group significantly increased with a positive and/or dose-related trend, meaning that the systemic toxicity of the Ag nanoparticles, including liver and kidney toxicity, might be explained by extensive systemic distribution of Ag originating from the Ag nanoparticles. Our current results suggest that further study is required to identify that Ag detected outside the gastrointestinal tract were indeed a nanoparticle form or ionized form. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: nanoparticle; silicon dioxide; silver; iron oxide; toxicity; subchronic; biodistribution

Introduction

Particles between 1 and 100 nm in size are called nanoparticles (NSET, 2010). Nanotechnology is one of the most rapidly developing fields in the history of humankind because nanomaterials have unique size-dependent physicochemical properties compared with bulk chemicals (Maynard *et al*., 2006). Recently, a broad range of nanomaterials have been developed for wide-ranging applications in all industrial and public sectors, including food, healthcare, agriculture, transport, energy, materials, information and communication technologies (Chun, 2009; Donaldson *et al*., 2004; Hristozov *et al*., 2012; Lux-Research, 2008; Rashidi and Khosravi-Darani, 2011). As the possibility of much broader public exposure of nanomaterials to the human body increases, safety concerns regarding nanoparticles are one of the most important issues.

The toxicities of the nanoparticles largely depend upon their several properties, including size, concentration, morphology, structure and solubility of particles (Cho *et al*., 2013a; Fubini *et al*., 2010; Maynard *et al*., 2011). In particular, smaller particles which have a large gross surface area are known to be more toxic, indicating that their large surface area is responsible for the greater

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hazard of the nanoparticles (Cho *et al.*, 2013a; Duffin *et al.*, 2007; Wittmaack, 2007). In fact, the nanoparticles are able to enter the body through the lung and intestinal tract more than microparticles due to their size and surface modifications (Jani *et al.*, 1990; Jia, 2005; Sigfridsson *et al.*, 2009). In case of the skin penetration of particles, size is generally regarded to play an important role (Schneider *et al.*, 2009; Schramlová *et al.*, 1997) although study using TiO_2 nanoparticles showed that physicochemical parameters, including size, shape and surface characteristics, did not affect the skin penetration pattern of the nanoparticles (Gamer *et al.*, 2006). These provide evidence that the absorption rate of particles is another important factor involved in a risk perspective (Cho *et al.*, 2013b).

There are various kinds of nanoparticles, such as silicon dioxide (SiO_2), silver (Ag), iron oxide (Fe_2O_3), titanium dioxide (TiO_2) and zinc oxide (ZnO). The applications of SiO_2 nanoparticles in industry, biomedicine, food and environmental protection are extremely promising because they possess extraordinary properties, such as good stabilities and excellent biocompatibilities and easy modifications (Xu *et al.*, 2014). In addition, the SiO_2 nanoparticles have great potential for wide applications in controllable drug delivery (Li *et al.*, 2012), medical diagnosis (Chen *et al.*, 2012), *in vivo* imaging (Bonacchi *et al.*, 2011; Shiohara *et al.*, 2010; Tu *et al.*, 2010), biosensor (Zhang *et al.*, 2004), photocatalysis (Badr *et al.*, 2008) and degradation of toxicants (Dong *et al.*, 2012; Saxena *et al.*, 2012; Singh *et al.*, 2009). The Ag nanoparticles are the most commonly used engineered nanomaterials in a variety of medical and consumer products associated with their antibacterial activity (SCENIHR, 2014; Vandebriel *et al.*, 2014; Wijnhoven *et al.*, 2009). In addition, the Fe_2O_3 nanoparticles are used in construction, paints/coating, plastics, cosmetics and nutriments (Ban *et al.*, 2013).

The information on *in vivo* kinetics of the nanoparticles is essential to understand their hazard by providing the actual concentration of the nanoparticles as they interact with biological systems (Cho *et al.*, 2013b). Despite the difference of kinetic and subsequent toxicity of the nanoparticles depending on the route of exposure, the effects of oral dosing of the nanoparticles compared with other routes of administration remain unclear. We recently reported the biodistribution and toxicity of the TiO_2 or ZnO nanoparticles after repeated oral administration (Cho *et al.*, 2013b; Seok *et al.*, 2013). In the current study, we performed the subchronic oral study to identify the systemic toxicity of the SiO_2, Ag and Fe_2O_3 nanoparticles to investigate the potential hazards and safety concerns associated with the nanoparticles.

Materials and Methods

Nanoparticles

Well-dispersed SiO_2 and citrate-capped Ag nanoparticles at 20 wt% in distilled water (DW) were provided by ABC Nanotech Co., Ltd. (Daejeon, Korea). In addition, Fe_2O_3 nanoparticles in powder form were obtained from NanoAmor Co., Ltd. (Houston, TX, USA). Primary sizes and morphology of the nanoparticles were measured via scanning electron microscopy (SEM, S-3500N; Hitachi Science Systems, Ltd., Ibaraki, Japan) and transmission electron microscopy (TEM, LEO-912 AB Omega; LEO, Tokyo, Japan). Dynamic light scattering (DLS) is often employed to monitor aggregation of nanoparticles (Afrooz *et al.*, 2013). The measurement of hydrodynamic size and zeta potential of the nanoparticles in DW using DLS was determined with a Delsa Nano (Beckman Coulter, Inc., Fullerton, CA, USA) according to the manufacturer's instructions.

Animals

Five-week-old female Sprague–Dawley rats were obtained from Orient Bio (Seongnam, Korea) and were housed in temperature- and light-controlled animal facilities, and were allowed to have access to a rodent diet (Teklad Certified Irradiated Global 18% Protein Rodent Diet; Harlan Laboratories, Madison, WI, USA) with tap water ad libitum. All of the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital. In addition, all of the animal experimental procedures were carried out by following the Organization for Economic Co-operation and Development (OECD) test guidelines as well as the Good Laboratory Practices for toxicity test guidance issued by the Korea Food and Drug Administration (KFDA, 2005).

Experimental Designs

The acute oral toxicity study was conducted as preliminary reference for dose selection in accordance with the OECD test guideline 420. In the situation where there is little or no information about the toxicity of the test material, a limit dose (2000 mg kg^{-1} body weight) can be carried out for acute studies (OECD, 2001). Therefore, the Fe_2O_3 nanoparticles were orally administered to Sprague–Dawley rats (five per sex per group) at a single dose of 2000 mg kg^{-1} of body weight. For the preparation of the Fe_2O_3 nanoparticles, 2000 mg of Fe_2O_3 powder was mixed with 98 ml of DW, followed by blending with the 0.2% citric acid and 0.9% sodium citrate at pH 5.5 to form the Fe_2O_3 aqueous suspension. The dispersion was treated by using an ultrasonic processor (VCX-750; Sonics & Materials Inc., Newtown, CT, USA) for 10 min. After carboxymethyl cellulose was added to a final concentration of 0.05%, the solution of the Fe_2O_3 nanoparticles was concentrated under vacuum to a final concentration of 20%. As the SiO_2 and Ag nanoparticles dispersed in DW were provided from the manufacturer, we first analyzed the amount of nanoparticles in DW. In addition, the SiO_2 and Ag nanoparticles were orally administered to Sprague–Dawley rats (five per sex per group) at a single dose of 1959 and 2061 mg kg^{-1} body weight, respectively. Clinical observations and mortality checks were conducted once per hour for 6 h after dosing and once daily thereafter for 14 days. Body weights were measured on the day of treatment and on test days 1, 7 and 14. At the end of the study, all of the animals were anesthetized with isoflurane, and whole blood was collected from anesthetized animals via the posterior vena cava.

The preliminary 14-day repeated oral toxicity study was also conducted to select the nanoparticle dose levels used in the 13-week repeated oral toxicity study. The SiO_2 nanoparticles were orally administered to Sprague–Dawley rats (five per sex per group) with doses of 489.8, 979.5 and 1959 mg kg^{-1}, the Ag nanoparticles were administered with doses of 515.3, 1030.5 and 2061 mg kg^{-1} and the Fe_2O_3 nanoparticles were treated with doses of 500, 1000 and 2000 mg kg^{-1} daily for 14 days. During the study period, the clinical signs and mortality of the rats were observed daily and the body weights were measured twice a week. The rats were anesthetized with isoflurane 1 day after the final gavage, and blood was taken via the posterior vena cava.

The 13-week repeated oral toxicity study was performed according to the OECD test guideline 408. Doses for the subchronic studies were based on results of the 14-day toxicity studies. However, a limit dose (1000 mg kg^{-1}) may be used for the high-dose level for subchronic studies if no toxicity is found (OECD, 1998). Therefore, the SiO_2 nanoparticles were orally administered to Sprague–

Dawley rats (12 per sex per group) with doses of 244.9, 489.8 and 979.5 mg kg^{-1}, the Ag nanoparticles were administered with doses of 257.6, 515.3 and 1030.5 mg kg^{-1} and the Fe_2O_3 nanoparticles were treated with doses of 250, 500 and 1000 mg kg^{-1} daily for 13 weeks. During the study period, the clinical signs and mortality of the rats were observed daily and the body weights were recorded weekly. The rats were anesthetized with isoflurane 1 day after the final gavage, and blood was taken via the posterior vena cava.

Urinalysis, Ophthalmoscope Examination, Hematology and Serum Biochemistry

During the last week of treatment, a urine analyzer (Miditron Junior II; Roche, Mannheim, Germany) was used according to the manufacturer's instructions to perform a urinalysis of 10 rats per group (five males and five females) by using fresh urine to determine the pH, specific gravity, leukocyte, nitrite, protein, ketone body, urobilinogen, bilirubin, glucose and hemoglobin. The external eye, ocular fundus, conjunctiva, sclera, cornea, lens and iris were examined using an ophthalmoscope.

Whole blood samples were collected into an EDTA blood collection tube and were applied to an automatic hematology analyzer ADVIA 2120i (Siemens Diagnostics, Tarrytown, NY, USA) for measurement of the following parameters: total white blood cell (WBC), red blood cell, hemoglobin, hematocrit, platelet, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and differential WBC.

For the serum biochemistry analysis, whole blood was centrifuged at 3000 rpm (2095 g) for 15 min, and serum was immediately separated and stored at –80 °C before analysis. The following serum biochemistry parameters were evaluated using an automatic chemistry analyzer 7070 (Hitachi, Tokyo, Japan) according to the instructions provided by the manufacturer: blood urea nitrogen, creatinine, total cholesterol, total protein, albumin, A/G ratio, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase, alanine transaminase, γ-glutamyl transferase, triglyceride, glucose, K, Na, Ca, P and Cl.

Gross Findings, Organ Weights and Histopathological Assessment

During necropsy, the heart, liver, lung, spleen, thymus, kidney, adrenal gland, testis, ovary, brain and pituitary gland were removed, weighed and fixed in 10% neutral formalin, except for testis and epididymis, which were fixed in Bouin's solution, and the eyes with the Harderian glands, which were fixed in Davidson solution (30 ml 95% ethyl alcohol + 20 ml formalin + 10 ml glacial acetic acid + 30 ml DW). The nasal cavity, spinal cords with bones, sternum and femora were treated with a decalcification solution for up to 3 weeks. Tissue slices were routinely processed for paraffin embedding, sectioning, hematoxylin and eosin staining. The histopathological changes were examined via light microscopy.

Distribution and Excretion of Si, Ag and Fe Following Repeated Oral Nanoparticle Administration for 13 Weeks

To identify the relationship between the nanoparticle distribution and the nanoparticle-induced systemic toxicity, the Si, Ag and Fe concentrations in sampled blood, organs, urine and feces were measured after repeated oral administration of the nanoparticles. For this, the samples from the blood, liver, kidney, spleen, lung and brain from five rats per group were obtained and weighed. In addition, the urine and feces were obtained using a metabolic cage for 24 h after the gavage to evaluate the nanoparticle excretion. All of the samples were analyzed for elemental Si, Ag or Fe to represent the nanoparticles with an inductively coupled plasma mass spectrometer (X7; Thermo Elemental, Winsford, Cheshire, UK) as previously described on the distributions of the TiO_2, ZnO and gold nanoparticles (Cho *et al.*, 2010, 2013b).

Statistical Analysis

All of the data were expressed as means ± SD. The acute toxicity study data were analyzed using Student's *t*-test. In addition, the data of the subchronic toxicity study were analyzed by using a one-way ANOVA with the SPSS software version 19 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant. If the variance was significant, the data were analyzed by the multiple comparison procedure of Dunnett's test.

Results

Nanoparticle Characterization

Images of the SiO_2, Ag and Fe_2O_3 nanoparticles using SEM and TEM were spherical, non-agglomerated and uniform (Fig. 1). Table 1 summarizes the physicochemical properties of the SiO_2, Ag and Fe_2O_3 nanoparticles. The primary sizes of the SiO_2, Ag

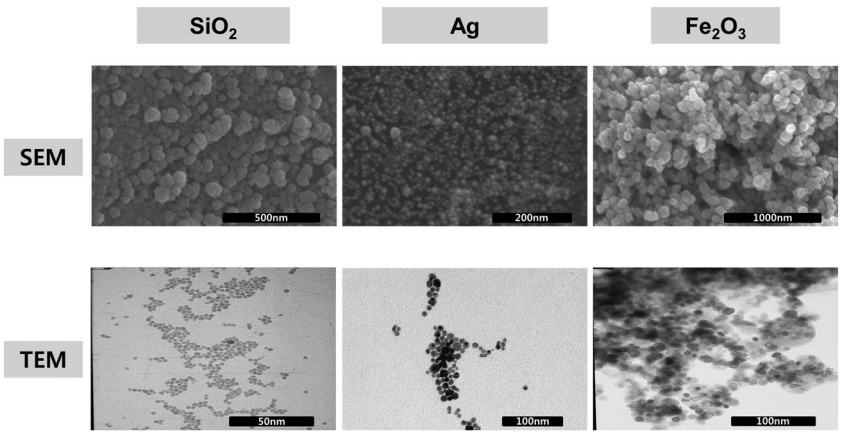

Figure 1. SEM and TEM of the SiO_2, Ag and Fe_2O_3 nanoparticles. Images of the SiO_2, Ag, and Fe_2O_3 nanoparticles show spherical shape. SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Table 1. Physicochemical characterization of the nanoparticles

Particles	Crystallinity	Primary size (nm)[a]	Hydrodynamic size (nm) in aqueous suspension	Zeta potential (mV)[d]
SiO_2	Spherical	12	33.5 ± 7.5[b]	–44.37
Ag	Spherical	11	19.0 ± 4.6[b]	–21.13
Fe_2O_3	α form	60	117.9 ± 78.0[b] (140.4 ± 69.3)[c]	13.60

DLS, dynamic light scattering; DW, distilled water.
[a]Primary nanoparticle sizes were measured by transmission electron microscopy.
Hydrodynamic sizes of nanoparticles in DW[b] or 0.05% carboxymethyl cellulose solution[c] were measured by the DLS method. The SiO_2 and Ag nanoparticles were dispersed in DW. The Fe_2O_3 nanoparticles were dispersed in DW and 0.05% carboxymethyl cellulose solution.
[d]Electrical stability of nanoparticle suspensions (pH 6.0) were determined by the DLS method.

and Fe_2O_3 nanoparticles measured by TEM were approximately 12 nm, 11 nm and 60 nm in diameter, respectively. The hydrodynamic size of the nanoparticles was measured by DLS to monitor aggregation of nanoparticles, as used in our previous study (Cho *et al.*, 2013b). DLS measurements showed that the average size of the SiO_2, Ag and Fe_2O_3 nanoparticles are 33.5 ± 7.5, 19.0 ± 4.6 and 117.9 ± 78.0 nm in diameter, respectively, suggesting that the SiO_2 and Fe_2O_3 nanoparticles formed small aggregates when dispersed in aqueous solution. The Ag nanoparticles had sizes similar to the primary size. The zeta potential of the SiO_2 nanoparticles was –44.37 mV for pH 6. In addition, the zeta potential of the Ag and Fe_2O_3 nanoparticles were –21.13 mV and 13.60 mV for pH 6, respectively.

Acute Oral Toxicity and 14-day Repeated Oral Toxicity Study

A single dose acute toxicity study and a 14-day repeated toxicity study were carried out to select the treatment doses of the nanoparticles in the subchronic toxicity study. In the acute oral toxicity study, doses of the 1959, 2061 and 2000 mg kg^{-1} of body weight were used for the SiO_2, Ag and Fe_2O_3 nanoparticles, respectively, and no mortalities were observed in both the vehicle control rats and the nanoparticle-treated rats. The nanoparticle groups also showed no treatment-related changes in body weights (Supplementary Fig. 1). In addition, necropsy findings also revealed no treatment-related effects, indicating that the acute oral LD_{50} in rats for the SiO_2, Ag and Fe_2O_3 nanoparticles have been estimated to be greater than 1959, 2061 and 2000 mg kg^{-1} of body weight, respectively.

In the 14-day repeated oral toxicity study, there were no significant dose-related changes in terms of mortality, clinical observations, body weight, hematology, serum biochemistry and organ weights and histopathology after the oral exposure to the SiO_2 and Fe_2O_3 nanoparticles in males and females for all of the test groups (data not shown). Meanwhile, the ALP levels significantly increased in males treated with 1030.5 (333.2 ± 71.2) and 2061 mg kg^{-1} (400.6 ± 85.8) of the Ag nanoparticles and in females treated with 515.3 (216.3 ± 60.1), 1030.5 (196.8 ± 33.6) and 2061 mg kg^{-1} (233.2 ± 29.2) of the Ag nanoparticles in comparison to those of the respective control groups (male control, 204.4 ± 28.9; female control, 120.2 ± 25.2). However, all of other parameters, including body weight, hematology, clinical observation and organ weight, showed no dose-related changes after oral administration of the Ag nanoparticles (data not shown). The results of the acute toxicity study and the 14-day repeated toxicity study indicated that no apparent toxic effects of the SiO_2, Ag and Fe_2O_3 nanoparticles was found, leading that 975.9, 1030.5 and 1000 mg kg^{-1} were selected as the highest dose of the SiO_2, Ag and Fe_2O_3 nanoparticles for the 13-week repeated toxicity study according to the OECD test guidelines.

Changes in Body Weight, Daily Feed Intake and Water Consumption in Subchronic Oral Toxicity

To identify the effects of subchronic oral dosing of the nanoparticles, the rats were gavaged with nanoparticles daily for 13 weeks. During the full period of the experiment, there was no significant difference in body weight between the nanoparticle groups and the respective control groups in both males and females (Fig. 2). In addition, the SiO_2, Ag and Fe_2O_3 nanoparticles had no significant effects on mean daily food and water consumption in either sex of all test groups (Supplementary Fig. 2).

Urinalysis, Ophthalmoscope Examination, Hematology and Clinical Chemistry in Subchronic Oral Toxicity

During the urinalysis and ophthalmoscope examination, there were no significant dose-associated adverse effects after the SiO_2, Ag and Fe_2O_3 nanoparticle administrations (data not shown).

Hematology revealed that the WBC level in females treated with 1030.5 mg kg^{-1} of the Ag nanoparticles was significantly higher than that in control group. In addition, the platelet level in males treated with 1030.5 mg kg^{-1} of the Ag nanoparticles was significantly lower than that in the control group (Table 2). On the other hand, the SiO_2 and Fe_2O_3 nanoparticle groups in the hematological parameters did not show significant changes relative to those of the respective control groups (Supplementary Tables 1 and 2).

The biochemical analysis of the serum indicated that the ALP level at 1030.5 mg kg^{-1} in the Ag nanoparticle-treated group significantly increased relative to that in the control group in both males and females. The calcium levels in all the dose groups treated with the Ag nanoparticles were significantly higher than in the control group in females (Table 3). The changes of serum biochemical parameters in the SiO_2 and Fe_2O_3 nanoparticle groups were sporadic and were of a small magnitude (Supplementary Tables 3 and 4), indicating that these differences were not considered dose-related adverse effects of the nanoparticle treatments.

Clinical Observation, Organ Weights and Histopathological Changes in Subchronic Oral Toxicity

During the entire exposure periods, no abnormal daily activity and clinical symptoms were observed in the SiO_2, Ag and Fe_2O_3 nanoparticle groups. The organs of rats evaluated via gross visual observation in the SiO_2, Ag and Fe_2O_3 nanoparticle groups appeared similar to controls.

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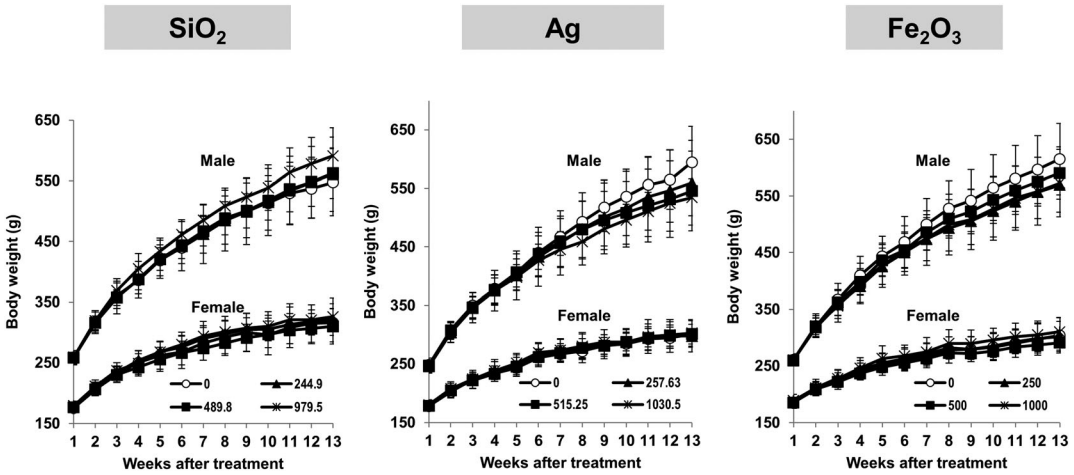

Figure 2. Growth curves for Sprague–Dawley rats orally administered with the SiO_2, Ag and Fe_2O_3 nanoparticles for 13 weeks. Body weights of Sprague–Dawley rats following the treatment of nanoparticles (mg kg^{-1}) were measured weekly during the study period. Data expressed as means ± SD.

Table 2. Hematological values for Sprague–Dawley rats orally administered with Ag nanoparticles for 13 weeks

	Ag (n = 12 per group)			
	0 mg kg^{-1}[a]	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}
Males				
WBC (10^3 mm^{-3})	7.8 ± 1.9[b]	6.7 ± 1.9	8.2 ± 1.0	8.7 ± 2.3
RBC (10^6 mm^{-3})	7.5 ± 0.4	7.4 ± 0.5	7.6 ± 0.3	7.5 ± 0.3
HGB (g dl^{-1})	14.1 ± 0.5	13.9 ± 0.9	14.1 ± 0.5	14.4 ± 0.4
HCT (%)	36.9 ± 1.3	37.0 ± 2.9	37.0 ± 1.9	38.2 ± 1.5
PLT (10^3 mm^{-3})	685 ± 78	705 ± 65	748 ± 80	563 ± 193*
MCV (fl)	49.3 ± 1.5	50.2 ± 2.5	48.7 ± 2.0	50.7 ± 1.4
MCH (pg)	18.8 ± 0.6	18.8 ± 0.5	18.5 ± 0.5	19.1 ± 0.8
MCHC (g dl^{-1})	38.2 ± 0.6	37.5 ± 1.6	38.0 ± 1.0	37.7 ± 1.1
Neutrophils (%)	13.6 ± 5.8	11.8 ± 3.9	12.2 ± 3.5	12.5 ± 3.6
Eosinophils (%)	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.1
Basophils (%)	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.6 ± 0.2
Lymphocytes (%)	79.4 ± 7.4	81.2 ± 4.7	80.8 ± 4.4	80.4 ± 4.6
Monocytes (%)	4.7 ± 1.4	4.6 ± 0.9	4.7 ± 0.9	4.9 ± 1.3
Reticulocyte (%)	21.0 ± 0.7	20.3 ± 0.7	19.0 ± 6.0	21.1 ± 0.9
Females				
WBC (10^3 mm^{-3})	5.7 ± 1.2	5.1 ± 1.4	5.7 ± 1.6	7.4 ± 2.4*
RBC (10^6 mm^{-3})	6.5 ± 0.4	6.5 ± 0.4	6.7 ± 0.3	6.6 ± 0.2
HGB (g dl^{-1})	13.1 ± 0.4	13.3 ± 0.3	13.3 ± 0.4	13.3 ± 0.3
HCT (%)	33.7 ± 1.7	34.2 ± 1.2	34.6 ± 1.2	34.0 ± 1.2
PLT (10^3 mm^{-3})	677 ± 69	610 ± 82	672 ± 63	751 ± 323
MCV (fl)	52.2 ± 1.8	52.5 ± 2.4	51.8 ± 2.1	51.4 ± 1.4
MCH (pg)	20.3 ± 0.8	20.4 ± 1.1	19.9 ± 0.9	20.1 ± 0.7
MCHC (g dl^{-1})	38.9 ± 1.3	38.8 ± 1.5	38.5 ± 1.0	39.1 ± 0.9
Neutrophils (%)	10.2 ± 3.0	9.7 ± 2.4	10.7 ± 2.9	12.2 ± 4.1
Eosinophils (%)	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
Basophils (%)	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
Lymphocytes (%)	83.6 ± 3.9	84.1 ± 3.0	83.0 ± 3.2	81.1 ± 5.1
Monocytes (%)	4.0 ± 0.8	4.0 ± 0.7	4.0 ± 0.7	4.5 ± 0.9
Reticulocyte (%)	22.8 ± 0.9	21.9 ± 0.7*	22.5 ± 1.1	21.7 ± 0.7*

HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

[a]Control group.

[b]Data expressed as means ± SD (n = 12 per group).

* Significantly different from the control group ($P < 0.05$).

Table 3. Serum biochemistry data for Sprague–Dawley rats orally administered with Ag nanoparticles for 13 weeks

	Ag (n = 12 per group)			
	0 mg kg^{-1}[a]	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}
Males				
BUN (mg dl^{-1})	14.5 ± 2.0[b]	15.0 ± 2.0	15.0 ± 0.9	14.8 ± 2.0
TC (mg dl^{-1})	55.2 ± 10.6	71.7 ± 25.3	74.2 ± 22.8	70.7 ± 18.2
TP (g dl^{-1})	5.7 ± 0.3	5.9 ± 0.3	5.9 ± 0.6	5.8 ± 0.4
Albumin (g dl^{-1})	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	2.2 ± 0.1
A/G	0.17 ± 0.39	0.33 ± 0.65	0.36 ± 0.50	0.33 ± 0.49
TB (mg dl^{-1})	0.00 ± 0.00	0.01 ± 0.03	0.00 ± 0.00	0.02 ± 0.04
ALP (IU l^{-1})	87.7 ± 19.8	102.2 ± 28.3	94.8 ± 21.9	123.3 ± 51.8*
AST (IU l^{-1})	95.7 ± 24.6	113.0 ± 44.8	86.9 ± 18.0	107.8 ± 84.2
ALT (IU l^{-1})	42.1 ± 9.9	50.7 ± 27.1	41.5 ± 10.5	58.1 ± 43.6
γGT (IU l^{-1})	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0
Creatinine (mg dl^{-1})	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
TG (mg dl^{-1})	98.8 ± 80.0	63.7 ± 34.7	78.8 ± 30.6	55.6 ± 24.7
Glucose (mg l^{-1})	164.0 ± 23.9	154.6 ± 18.2	158.7 ± 9.8	154.0 ± 10.8
K (mmol l^{-1})	4.6 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	4.4 ± 0.3
Na (mmol l^{-1})	142.3 ± 2.5	143.1 ± 2.3	144.2 ± 2.3	144.4 ± 2.5
Ca (mg dl^{-1})	9.2 ± 0.5	9.6 ± 0.4	9.5 ± 0.6	9.4 ± 0.4
P (mg l^{-1})	7.0 ± 0.6	7.0 ± 0.5	6.8 ± 0.5	6.9 ± 0.4
Cl (mmol l^{-1})	106.0 ± 3.1	107.4 ± 2.7	106.7 ± 1.9	107.9 ± 2.5
Females				
BUN (mg dl^{-1})	13.8 ± 2.0	13.9 ± 2.2	14.0 ± 1.5	14.7 ± 3.1
TC (mg dl^{-1})	73.5 ± 20.3	84.6 ± 12.4	71.5 ± 9.1	78.9 ± 12.9
TP (g dl^{-1})	6.4 ± 0.5	6.3 ± 0.4	6.3 ± 0.4	6.1 ± 0.4
Albumin (g dl^{-1})	2.7 ± 0.3	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.2
A/G	0.17 ± 0.39	0.17 ± 0.39	0.33 ± 0.65	0.33 ± 0.65
TB (mg dl^{-1})	0.03 ± 0.05	0.03 ± 0.05	0.03 ± 0.05	0.02 ± 0.04
ALP (IU l^{-1})	40.0 ± 10.1	52.3 ± 17.0	54.1 ± 12.9	59.0 ± 21.8*
AST (IU l^{-1})	90.6 ± 27.9	86.9 ± 28.8	91.8 ± 13.8	91.1 ± 25.3
ALT (IU l^{-1})	38.3 ± 9.3	44.1 ± 13.1	41.4 ± 13.7	40.3 ± 14.0
γGT (IU l^{-1})	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1
Creatinine (mg dl^{-1})	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
TG (mg dl^{-1})	44.5 ± 34.4	31.3 ± 24.0	24.2 ± 9.9	27.0 ± 13.1
Glucose (mg l^{-1})	152.7 ± 14.4	165.0 ± 22.3	156.6 ± 24.4	155.3 ± 22.5
K (mmol l^{-1})	4.2 ± 0.2	4.0 ± 0.3	4.1 ± 0.3	4.2 ± 0.3
Na (mmol l^{-1})	140.4 ± 2.6	140.6 ± 2.4	143.0 ± 2.0	140.9 ± 4.1
Ca (mg dl^{-1})	9.8 ± 1.2	12.2 ± 1.4*	12.2 ± 1.3*	12.1 ± 1.2*
P (mg l^{-1})	5.6 ± 0.5	5.6 ± 0.6	5.9 ± 0.5	6.2 ± 0.7
Cl (mmol l^{-1})	102.3 ± 2.2	102.5 ± 3.1	102.3 ± 2.2	101.9 ± 3.1

A/G, albumin/globulin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; TB, total bilirubin; TC, total cholesterol; TG, triglyceride; TP, total protein; γGT, gamma glutamyl transferase.

[a]Control group.

[b]Data expressed as means ± SD (n = 12 per group).

* Significantly different from control group ($P < 0.05$).

A significant increase in absolute lung weight in males was found at high doses (979.5 mg kg^{-1}) in the SiO_2 nanoparticle treatment group. In males and females of all SiO_2 nanoparticle-treated groups, relative weights for all of the tested organs (g 1000 g^{-1} of body weight) were not significantly different compared to those in the respective control groups (Supplementary Table 5). In the Ag nanoparticle treatment group, the absolute pituitary gland weight significantly decreased at high doses (1030.5 mg kg^{-1}) in males. Meanwhile, the relative liver weight significantly decreased after the Ag nanoparticle treatment at a dose of 515.3 mg kg^{-1} in females. Significant increases in relative left ovary weight were observed at high doses (1030.5 mg kg^{-1}) in the Ag nanoparticle treatment group in females (Table 4). In the case of the Fe_2O_3 nanoparticles, the absolute pituitary gland weight significantly decreased in the high-dose group (1000 mg kg^{-1}) in female. The relative liver weight significantly decreased in the low-dose (250 mg kg^{-1}) and high-dose group (1000 mg kg^{-1}) in females. Significant increases in relative right adrenal gland weight were observed at high doses (1000 mg kg^{-1}) in the Fe_2O_3 nanoparticle treatment group in males. The relative testis weight significantly increased at low (250 mg kg^{-1}) and high doses (1000 mg kg^{-1}) of the Fe_2O_3 nanoparticles in males. In addition, the relative pituitary

Table 4. Final body and organ weights for male and female Sprague–Dawley rats orally administered with Ag nanoparticles for 13 weeks

		Ag (n = 12 per group)			
		0 mg kg^{-1}[a]	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}
Males					
Liver	(g)	16.78 ± 2.00[b]	16.34 ± 2.49	16.03 ± 3.22	15.11 ± 1.91
	(g 1000 g^{-1} BW)	29.51 ± 1.28	29.71 ± 1.56	29.61 ± 2.33	28.05 ± 2.57
Spleen	(g)	0.892 ± 0.124	0.887 ± 0.169	0.931 ± 0.237	1.017 ± 0.365
	(g 1000 g^{-1} BW)	1.581 ± 0.251	1.615 ± 0.239	1.723 ± 0.341	1.888 ± 0.660
Kidney (right)	(g)	1.840 ± 0.205	1.712 ± 0.232	1.669 ± 0.199	1.687 ± 0.149
	(g 1000 g^{-1} BW)	3.244 ± 0.230	3.127 ± 0.264	3.108 ± 0.157	3.144 ± 0.318
Kidney (left)	(g)	1.812 ± 0.206	1.729 ± 0.242	1.678 ± 0.209	1.666 ± 0.130
	(g 1000 g^{-1} BW)	3.193 ± 0.187	3.156 ± 0.286	3.125 ± 0.185	3.107 ± 0.298
Adrenal gland (right)	(g)	0.027 ± 0.005	0.029 ± 0.005	0.027 ± 0.003	0.029 ± 0.005
	(g 1000 g^{-1} BW)	0.047 ± 0.007	0.053 ± 0.010	0.050 ± 0.005	0.055 ± 0.010
Adrenal gland (left)	(g)	0.030 ± 0.005	0.029 ± 0.007	0.029 ± 0.003	0.031 ± 0.006
	(g 1000 g^{-1} BW)	0.052 ± 0.007	0.053 ± 0.013	0.053 ± 0.006	0.059 ± 0.012
Testis (right)	(g)	1.701 ± 0.165	1.609 ± 0.326	1.658 ± 0.098	1.721 ± 0.099
	(g 1000 g^{-1} BW)	3.023 ± 0.430	2.973 ± 0.732	3.118 ± 0.364	3.207 ± 0.216
Testis (left)	(g)	1.697 ± 0.144	1.615 ± 0.301	1.657 ± 0.116	1.720 ± 0.096
	(g 1000 g^{-1} BW)	3.015 ± 0.395	2.984 ± 0.690	3.115 ± 0.393	3.207 ± 0.234
Thymus	(g)	0.263 ± 0.086	0.272 ± 0.069	0.251 ± 0.060	0.247 ± 0.052
	(g 1000 g^{-1} BW)	0.457 ± 0.123	0.498 ± 0.128	0.466 ± 0.094	0.459 ± 0.095
Heart	(g)	1.644 ± 0.122	1.642 ± 0.172	1.604 ± 0.196	1.619 ± 0.110
	(g 1000 g^{-1} BW)	2.905 ± 0.173	3.006 ± 0.243	2.990 ± 0.247	3.019 ± 0.254
Lung	(g)	1.592 ± 0.126	1.642 ± 0.146	1.635 ± 0.236	1.593 ± 0.144
	(g 1000 g^{-1} BW)	2.820 ± 0.262	3.024 ± 0.380	3.052 ± 0.365	2.974 ± 0.353
Brain	(g)	2.168 ± 0.130	2.164 ± 0.153	2.111 ± 0.094	2.159 ± 0.089
	(g 1000 g^{-1} BW)	3.841 ± 0.304	3.988 ± 0.476	3.961 ± 0.339	4.026 ± 0.273
Pituitary gland	(g)	0.014 ± 0.001	0.014 ± 0.002	0.013 ± 0.001	0.013 ± 0.002*
	(g 1000 g^{-1} BW)	0.025 ± 0.002	0.025 ± 0.003	0.025 ± 0.004	0.023 ± 0.004
Females					
Liver	(g)	8.85 ± 1.10	8.68 ± 1.39	8.02 ± 0.66	8.20 ± 0.67
	(g 1000 g^{-1} BW)	29.93 ± 2.61	29.56 ± 3.20	27.30 ± 1.67*	27.73 ± 1.26
Spleen	(g)	0.615 ± 0.107	0.614 ± 0.113	0.599 ± 0.139	0.667 ± 0.131
	(g 1000 g^{-1} BW)	2.073 ± 0.240	2.100 ± 0.383	2.036 ± 0.436	2.256 ± 0.424
Kidney (right)	(g)	1.000 ± 0.075	0.970 ± 0.110	0.960 ± 0.113	0.974 ± 0.097
	(g 1000 g^{-1} BW)	3.387 ± 0.108	3.322 ± 0.345	3.267 ± 0.320	3.290 ± 0.215
Kidney (left)	(g)	0.982 ± 0.084	0.968 ± 0.106	0.926 ± 0.121	0.963 ± 0.084
	(g 1000 g^{-1} BW)	3.326 ± 0.166	3.315 ± 0.340	3.150 ± 0.353	3.259 ± 0.225
Adrenal gland (right)	(g)	0.033 ± 0.007	0.032 ± 0.003	0.034 ± 0.004	0.033 ± 0.005
	(g 1000 g^{-1} BW)	0.111 ± 0.022	0.111 ± 0.018	0.115 ± 0.013	0.113 ± 0.019
Adrenal gland (left)	(g)	0.034 ± 0.003	0.035 ± 0.005	0.037 ± 0.004	0.036 ± 0.005
	(g 1000 g^{-1} BW)	0.117 ± 0.014	0.120 ± 0.022	0.125 ± 0.011	0.124 ± 0.021
Ovary (right)	(g)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	(g 1000 g^{-1} BW)	0.20 ± 0.04	0.20 ± 0.03	0.19 ± 0.04	0.21 ± 0.03
Ovary (left)	(g)	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
	(g 1000 g^{-1} BW)	0.19 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	0.22 ± 0.03*
Thymus	(g)	0.272 ± 0.059	0.222 ± 0.048	0.220 ± 0.058	0.244 ± 0.065
	(g 1000 g^{-1} BW)	0.915 ± 0.163	0.764 ± 0.183	0.749 ± 0.189	0.825 ± 0.213
Heart	(g)	1.020 ± 0.089	0.979 ± 0.072	0.972 ± 0.078	1.005 ± 0.061
	(g 1000 g^{-1} BW)	3.459 ± 0.299	3.355 ± 0.228	3.310 ± 0.243	3.402 ± 0.148
Lung	(g)	1.202 ± 0.105	1.208 ± 0.117	1.169 ± 0.070	1.308 ± 0.194
	(g 1000 g^{-1} BW)	4.075 ± 0.298	4.148 ± 0.493	3.988 ± 0.289	4.452 ± 0.810
Brain	(g)	2.009 ± 0.124	2.020 ± 0.097	2.001 ± 0.075	1.987 ± 0.093
	(g 1000 g^{-1} BW)	6.828 ± 0.594	6.941 ± 0.612	6.835 ± 0.501	6.750 ± 0.607
Pituitary gland	(g)	0.016 ± 0.003	0.017 ± 0.004	0.017 ± 0.002	0.020 ± 0.010
	(g 1000 g^{-1} BW)	0.055 ± 0.011	0.060 ± 0.014	0.056 ± 0.009	0.069 ± 0.034

[a]Control group.
[b]Data expressed as means ± SD (N = 12/group).
* Significantly different from control group ($P < 0.05$).

gland weight significantly decreased at high doses (1000 mg kg^{-1}) in the Fe_2O_3 nanoparticle treatment group in females (Supplementary Table 6).

In the sampled organs examined for the SiO_2 and Fe_2O_3 nanoparticles, spontaneous lesions in several tissues using histopathology were observed in the nanoparticle-treated groups and the respective control groups. However, most of these changes were sporadic without dose-dependent trends, indicating that these changes were not considered toxicologically relevant (Supplementary Tables 7 and 8). Noteworthy, in the Ag nanoparticle-treated animals, the incidence of lymphocyte infiltration in livers in the high-dose group (eight of 12, male; six of 12, female) was higher than that in the control group (five of 12, male; four of 12, female) although there was no difference in severity of lymphocyte infiltration between two groups. When the Ag nanoparticles were orally administered, lymphocyte infiltration in kidneys was noted in one, none, two and four of the control, low-, middle- and high-dose male rats, respectively, and none, none, one and three of the control, low-, middle- and high-dose female rats, respectively, indicating the Ag nanoparticle treatment-related effect (Table 5).

Si, Ag and Fe Distribution in Tissues

To identify the relationship between Si, Ag and Fe accumulation and the nanoparticle-induced systemic toxicity, Si, Ag and Fe concentrations in sampled blood, organs, urine and feces were measured after repeated oral administration of the nanoparticles using an inductively coupled plasma mass spectrometer as previously described in the distributions of the TiO_2, ZnO and gold nanoparticles (Cho *et al.*, 2010, 2013b). The Si and Fe concentrations in the blood of the SiO_2 and Fe_2O_3 nanoparticle-treated rats showed no significant increase compared with the respective control groups (Supplementary Figs. 2 and 3). In all tissues tested, including liver, kidney, spleen, lung and brain, the concentrations of the Si and Fe showed no dose-associated response in comparison to the respective control groups, even in the high-dose group. The Si and Fe concentrations in the urine of the SiO_2 and Fe_2O_3 nanoparticle-treated rats showed no significant differences compared with the control groups. In contrast, the concentration of Si in the feces in the middle dose (489.8 mg kg^{-1} day^{-1}) and high-dose group (979.5 mg kg^{-1} day^{-1}) of the SiO_2 nanoparticle-treated animals was significantly higher than that of the control group, suggesting that most of the SiO_2 nanoparticles were excreted via the feces. Although not statistically significant, the concentrations of Fe in the feces in the Fe_2O_3 nanoparticle-treated animals were found to be higher than that of the control groups. However, for accurate analysis on the distribution of Fe associated with the administration of the Fe_2O_3 nanoparticles, further analysis is required to confirm because Fe is an essential element, widely distributed in the body (Wang *et al.*, 2010).

The concentrations of the Ag in the blood of the Ag nanoparticle treatment group significantly increased in comparison to the control group. In addition, the Ag concentration in the sampled organs showed obvious increases in all the dose groups treated with the Ag nanoparticles. In particular, increases in the Ag concentrations in spleen, lung and brain induced by the Ag nanoparticle administration showed a clear dose–response relationship in both male and female rats. Data on the excretion of the Ag nanoparticles was consistent with the absorption and distribution patterns. The urine in the high-dose group (1030.5 mg kg^{-1} day^{-1}) of the Ag nanoparticle-treated animals contains higher amounts of the Ag, but it did not reach statistical significance. In particular, the Ag concentration in the feces of the Ag nanoparticle treatment groups was significantly higher than that in the control group. The Ag concentration was detected more in the feces than those in the urine or tissues, suggesting that most of the nanoparticles were excreted via the feces (Fig. 3).

Discussion

Manufacturing industries are rapidly exploring potential applications of nanotechnology, and many consumer goods with engineered nanoparticles are entering the marketplace. In parallel, there are growing concerns about unanticipated adverse effects on the safety of the nanoparticles (Li *et al.*, 2010; Maynard *et al.*, 2006; Teeguarden *et al.*, 2008; Warheit, 2001). In general, a low absorption of particles in the body would be favorable from a risk perspective (Cho *et al.*, 2013b). However, the nanoparticles often show faster absorption into the blood via the respiratory and gastrointestinal systems than larger particles, which are largely dependent on the size and surface properties of particles (Fubini *et al.*, 2010; Lee *et al.*, 2012). This led us to address the potential risks of the nanoparticles to human health.

There are various kinds of nanoparticles, such as SiO_2, Ag and Fe_2O_3, which are widely used in various research and industry fields. Along with increased use of the SiO_2 nanoparticles, their exposure has been rising steadily, leading to more attention being paid to their potential risk to human health (Fu *et al.*, 2013; Lin and Haynes, 2010; Nel *et al.*, 2006; Yu *et al.*, 2011; Zhang *et al.*, 2012). Several studies have reported that the SiO_2 nanoparticles were found more toxic than the microparticles to the male reproductive systems (Fan *et al.*, 2006) with decreased number and motility rate of sperm and induction of the apoptosis of testicle spermatogenic cells (Lin *et al.*, 2007). Nonetheless, the SiO_2 nanoparticles are generally regarded as safe and have a wide variety of applications in industry, including cosmetic and food ingredients (Bonacchi *et al.*, 2011; Lee *et al.*, 2011; Napierska *et al.*, 2009). Recently, the toxicity of the Ag nanoparticles has become one of the most studied areas in the field of nanotoxicology. However, the toxic effect of the Ag nanoparticles has not been conclusively established. Even though several repeated dose exposure studies did not show severe systemic toxicity of the Ag nanoparticles probably because of the relatively low absorption through systemic routes, liver toxicity and bile duct hyperplasia were seen (Ji *et al.*, 2007; Kim *et al.*, 2008, 2010; Lee *et al.*, 2013; Sung *et al.*, 2009; Van Der Zande *et al.*, 2012). The Fe_2O_3 nanoparticles, which are used in construction, paints/coating, plastics, cosmetics and nutriments (Ban *et al.*, 2013), induced lung injury in mice (Ban *et al.*, 2012; Zhu *et al.*, 2008) and cytotoxic effects *in vitro* (Soto *et al.*, 2007).

The nanoparticles administered orally can be absorbed into the circulation via the site of entry. After administration, they are immobilized within the submucosal layer of the stomach and Peyer's patches, and then transported into the serosal layer and systemic circulation (Eldridge *et al.*, 1990). Compared with inhalation or skin exposure, the oral intake of the nanoparticles has the potential for wide exposure of the public to higher doses and more frequent ingestion (Chun, 2009; Rashidi and Khosravi-Darani, 2011). However, limited data have been reported for the nanoparticle exposure via the oral route. To identify the toxicity of the oral dosing of three important nanoparticles, SiO_2, Ag and Fe_2O_3, we performed the acute and subchronic oral toxicity studies in rats.

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Table 5. Histopathological changes in Sprague–Dawley rats orally administered with Ag nanoparticles for 13 weeks

	Ag (n = 12 per group)							
	Male				Female			
	0 mg kg^{-1}[a]	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}	0 mg kg^{-1}	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}
Esophagus	0[b]	0	0	0	0	0	0	0
Large intestine	0	0	0	0	0	0	0	0
Liver								
Fatty change	3	1	2	1	2	1	2	3
Lymphocyte infiltration	5	7	6	8	4	4	3	6
Pancreas								
Inflammatory cells around islet	1	1	2	0	0	0	0	0
Salivary gland	0	0	0	0	0	0	0	0
Small intestine	0	0	0	0	0	0	0	0
Stomach	0	0	0	0	0	0	0	0
Tongue	0	0	0	0	0	0	0	0
Heart								
Macrophage infiltration	1	1	2	0	1	1	0	0
Adrenal gland	0	0	0	0	0	0	0	0
Parathyroid gland	0	0	0	0	0	0	0	0
Pituitary gland	0	0	0	0	0	0	0	0
Thyroid gland	0	0	0	0	0	0	0	0
Epididymis	0	0	0	0	–	–	–	–
Preputial gland	0	0	0	0	–	–	–	–
Prostate	0	0	0	0	–	–	–	–
Seminal vesicle	0	0	0	0	–	–	–	–
Testis (testicular atrophy)	0	1	0	0	–	–	–	–
Cervix	–	–	–	–	0	0	0	0
Clitoral gland	–	–	–	–	0	0	0	0
Ovary								
Calcification	–	–	–	–	1	0	0	0
Hemorrhagic corpus luteum	–	–	–	–	0	0	1	00
Uterus	–	–	–	–	0	0	0	0
Bone marrow	0	0	0	0	0	0	0	0
Lymph node, mandibular								
Chronic inflammation in adjacent tissue	1	0	0	0	0	0	0	0
Lymph node, mesenteric	0	0	0	0	0	0	0	0
Spleen	0	0	0	0	0	0	0	0
Thymus	0	0	0	0	0	0	0	0
Mammary gland	0	0	0	0	0	0	0	0
Skin	0	0	0	0	0	0	0	0

(Continues)

Table 5. (Continued)

	Ag (n = 12 per group)							
	Male				Female			
	0 mg kg^{-1}[a]	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}	0 mg kg^{-1}	257.63 mg kg^{-1}	515.25 mg kg^{-1}	1030.5 mg kg^{-1}
Femur	0	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0
Sternum	0	0	0	0	0	0	0	0
Brain, nerve, spinal cord	0	0	0	0	0	0	0	0
Larynx	0	0	0	0	0	0	0	0
Lung								
Alveolar macrophage infiltration	0	0	1	0	0	0	0	0
Asphyxia	0	0	1	0	0	0	0	0
Aspiration pneumonia	0	1	1	1	0	1	0	3
Fibrotic nodule in subpleural area	0	0	0	0	0	1	0	0
Nasal cavity	0	0	0	0	0	0	0	0
Trachea								
Foreign body granuloma with abscess	1	0	0	0	0	0	0	0
Eye	0	0	0	0	0	0	0	0
Harderian gland (lymphocyte infiltration)	0	0	0	1	0	0	0	0
Kidney								
Lymphocyte infiltration	1	0	2	4	0	0	1	3
Calcification	0	0	0	0	7	7	10	9
Urinary bladder	0	0	0	0	0	0	0	0

[a]Control group.
[b]The number of animals with abnormal histopathological changes among 12 rats per each group.

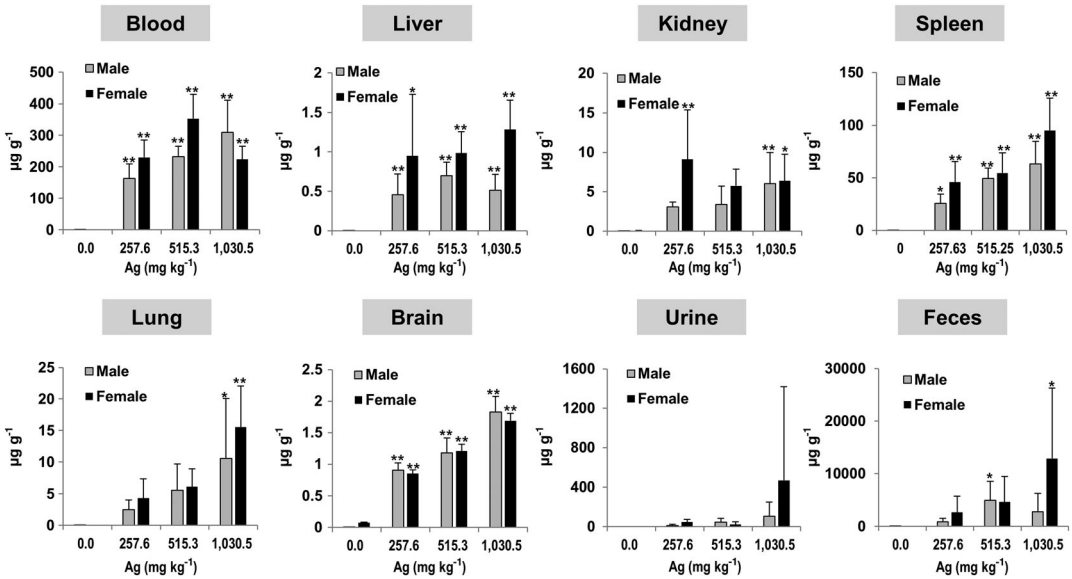

Figure 3. Concentration of the Ag in Sprague–Dawley rats orally administered with the Ag nanoparticles for 13 weeks. The concentrations of the Ag in the blood, liver, kidney, spleen, lung, brain, urine and feces following the treatment of the Ag nanoparticles (mg kg^{-1}) were evaluated using an inductively coupled plasma mass spectrometer. Data expressed as means ± SD. Significance versus control: $^{*}P < 0.05$, $^{**}P < 0.01$.

In the 13-week subchronic toxicity study, the rats treated with the SiO_2, Ag and Fe_2O_3 nanoparticles did not show any dose-associated differences in terms of body weight and food consumption following repeated oral dosing. In particular, our current results indicated that the rats with the SiO_2 and Fe_2O_3 nanoparticles showed no treatment-related changes in hematological, serum biochemical or histopathological lesions. On the other hand, the repeated oral administration of the Ag nanoparticles exerted the increase in incidence of lymphocyte infiltration in the liver, particularly portal inflammation, as evaluated by histopathology, along with the increase in WBC and serum ALP. This result was consistent with a previous study (Kim *et al.*, 2010) showing that the oral dosing of the Ag nanoparticles induced liver toxicity, including bile duct hyperplasia and increased foci. Interestingly, unlike the other report of Kim *et al.* (2010), serum calcium significantly increased in all the dose groups treated with the Ag nanoparticles in females in the current study. The mechanisms may have been involved, including mobilization of calcium from the bones or disturbance in renal excretion of calcium because of renal damage (Shull *et al.*, 1981). Elevated serum calcium was also linked to possible reduction in glomerular filtration (Bucci *et al.*, 1998). Actually, the oral treatment of the Ag nanoparticles induced lymphocyte infiltration in the kidney in the present study. Taken together, these results support that the Ag nanoparticles may exert potential toxic effects in the liver and kidney after the repeated oral dosing compared with the SiO_2 and Fe_2O_3 nanoparticles showing no systemic toxicity.

Oral uptake of nanoparticle food additives or ingredients is likely to occur at low doses over long periods. Nevertheless, limited reports on *in vivo* effects of nanoparticles, including the SiO_2, Ag and Fe_2O_3, upon subchronic oral administration are available (Dekkers *et al.*, 2013; EFSA, 2009). In an earlier study, the ZnO nanoparticles showed higher absorption and more extensive organ distribution compared with the TiO_2 nanoparticles after oral administration for 13 weeks (Cho *et al.*, 2013b). Consistently, the ZnO nanoparticles caused pancreatitis and anemia, probably because of the absorption of ionized Zn due to the complete dissolution of the ZnO nanoparticles (Seok *et al.*, 2013). Therefore, the present result on the Ag nanoparticle-induced systemic toxicity raises the possibility of the difference of biodistribution among the SiO_2, Ag and Fe_2O_3 nanoparticles. In particular, as the Ag nanoparticles induced slight inflammation in the liver and kidney, it can be speculated that uncleared Ag can be deposited in the kidney and liver. In this study, the Si and Fe concentrations in all tissues of the SiO_2 and Fe_2O_3 nanoparticle-treated rats showed no significant increase, whereas the Ag concentration in sampled blood and organs of the Ag nanoparticle-treated group significantly increased with a positive and/or dose-related trend although a large number of the Ag nanoparticles were excreted via the feces after gastrointestinal absorption. Therefore, we can conclude that the systemic toxicity of the Ag nanoparticles might be due to the tissue distribution of the Ag originating from the Ag nanoparticles. A more thorough study is necessary to provide evidence to identify that the Ag detected outside the gastrointestinal tract were indeed nanoparticle or ionized forms. In addition, further toxicity evaluation of the SiO_2 and Fe_2O_3 nanoparticles minimizing particle aggregation is also needed.

Acknowledgments

This research was supported by a grant (09152MFDS700, 13182MFDS606) from Ministry of Food and Drug Safety in 2009 and 2013.

Conflict of Interest

The authors did not report any conflict of interest.

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