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Cytotoxic Effects of Nanoparticles Assessed *In Vitro* and *In Vivo*

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Abstract An increasing number of applications is being developed for the use of nanoparticles in various fields. We investigated possible toxicities of nanoparticles in cell culture and in mice. Nanoparticles tested were Zn (300 nm), Fe (100 nm), and Si (10–20, 40–50, and 90–110 nm). The cell lines used were brain, liver, stomach, and lung from humans. In the presence of nanopaticles, mitochodrial activity decreased zero to 15%. DNA contents decreased zero to 20%, and glutathione production increased zero to 15%. None of them showed a dose dependency. Plasma membrane permeability was not altered by nanoparticles. In the case of Si, different sizes of the nanoparticles did not affect cytotoxicity. The cytotoxicity was also shown to be similar in the presence of micro-sized (45 μm) Si particles. Organs from mice fed with nanoparticles showed nonspecific hemorrhage, lymphocytic infiltration, and medullary congestion. A treatment with the micro-sized particle showed similar results, suggesting that the acute *in vivo* toxicity was not altered by nano-sized particles.

Keywords: Nanoparticles, cytotoxicity, *in vivo* toxicity, MTT assay, DNA contents, glutathione production, histochemical pathology test

Nanoparticles are those having diameters of nanometer size. With the advent of modern technology, humans can make nano-sized particles that were not present in nature. The use of nanotechnology extends to medicine, biotechnology, materials, process development, energy, and environments [4, 7, 11, 16, 17]. Nanomaterials used include nanotubes, nanowires, fullerene derivatives, and quantum dots. As the size of the particle is reduced, many new properties are shown in the fields of magnetics and semiconductivities. Since the nano-sized particles were not present in nature, and human exposure to such particles could pose a potential health problem, owing to the artificial production of them we wanted to measure any possible toxicity of nanoparticles both *in vitro* and *in vivo*. There have been several reports about the toxicity of nanomaterials [1–3, 5, 6, 8, 12, 14, 15]. The approach was either *in vitro* or *in vivo*, and no comparison was shown with micro-sized particles. Here, we report the investigation on the toxicity of three different nanoparticles along with a micro-sized paticle as a control. Moreover, the effect of size within the same nanoparticle was assessed.

The following human cell lines were used; liver (Huh-7), brain (A-172), stomach (MKN-1), lung (A-549), and kidney (HEK293). Cells were grown in DMEM (HyClone, U.S.A.) supplemented with 10% FBS (HyClone, U.S.A.) and 1% penicillin-streptomycin. For the cytotoxicity test, cells were treated with each nanoparticle at the concentration of 0.24, 2.4, 24, 240, and 2,400 ppb.

Nanoparticles used were Zn (300 nm), Fe (100 nm), and Si (10–20, 40–50, 90–110 nm). Micro-sized (45 μm) Si was also used as a control. All the particles were a kind gift from Professor Hee Kwon Chae.

For mitochondrial activity tests, cells were seeded at the concentration of 2.5×10^3 cells/well in a 96-well plate and incubated. Various concentrations of nanoparticles were added in 24 h. Cells were further incubated for 72 h before 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) was added to each well at the concentration of 0.5 mg/ml. After 3 h of incubation, the media were discarded and 50 ml of dimethyl sulfoxide (DMSO) was added to each well. Optical density was measured at 595 nm in 10 min at 60°C [7].

For DNA contents assay, cells were seeded in a 24-well plate at the concentration of 5×10^4 cells/well and incubated. Various concentrations of nanoparticles were added in 24 h. Cells were further incubated for 72 h and washed with phosphate-buffered saline (PBS, pH 7.4). Two-hundred ml of RIPA buffer containing 50 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 150 mM sodium chloride was added to each well and incubated on ice for 30 min. Each sample was collected in a tube and subject to centrifugation at 14,000 ×*g* for 10 min. Two μl of each lysed sample and 100 μl of bisbenzimide H 33258 solution (2 mg/ml) were mixed in a 96-well plate

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and fluorescence was measured (excitation at 360 nm, emission at 460 nm).

For glutathione production, cells were seeded in a 24-well plate at the concentration of 5×10^4 cells/well and incubated. Various concentrations of nanoparticles were added in 24 h. After 72 h incubation, they were washed with PBS and homogenized with 5% sulfosalicylic acid (SSA). Each homogenate was subjected to centrifugation at 14,000 $\times g$ in 4°C for 5 min. Fifteen µl of 100 U/ml glutathione reductase, 100 µl of NADPH, and 15 µl of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were added to each homogenate and incubated at 37°C for 10 min. Optical density was measured at 412 nm. Total protein concentration was measured using the Bradford assay.

For membrane permeability tests, cells were seeded in a 24-well plate at the concentration of 5×10^4 cells/well and incubated. Various concentrations of nanoparticles were added in 24 h. After 72 h incubation, they were washed with PBS and 200 ml of 2.5 mg/ml fluorescein isothiocyanate-dextran (Sigma, U.S.A.) was added and further incubated for 30 min. After washing with PBS, cells were fixed with 5% paraformaldehyde (Sigma, U.S.A.) and observed under fluorescent phase-contrast microscopy.

A 7-week-old Balb/c mouse (male) was starved for 24 h before feeding 2.5 g of each nanoparticle. Three days after feeding, the mouse was sacrificed and the heart, liver, spleen, stomach, and intestine were taken and subjected to histopathological observation [9, 10, 13]. Each organ was

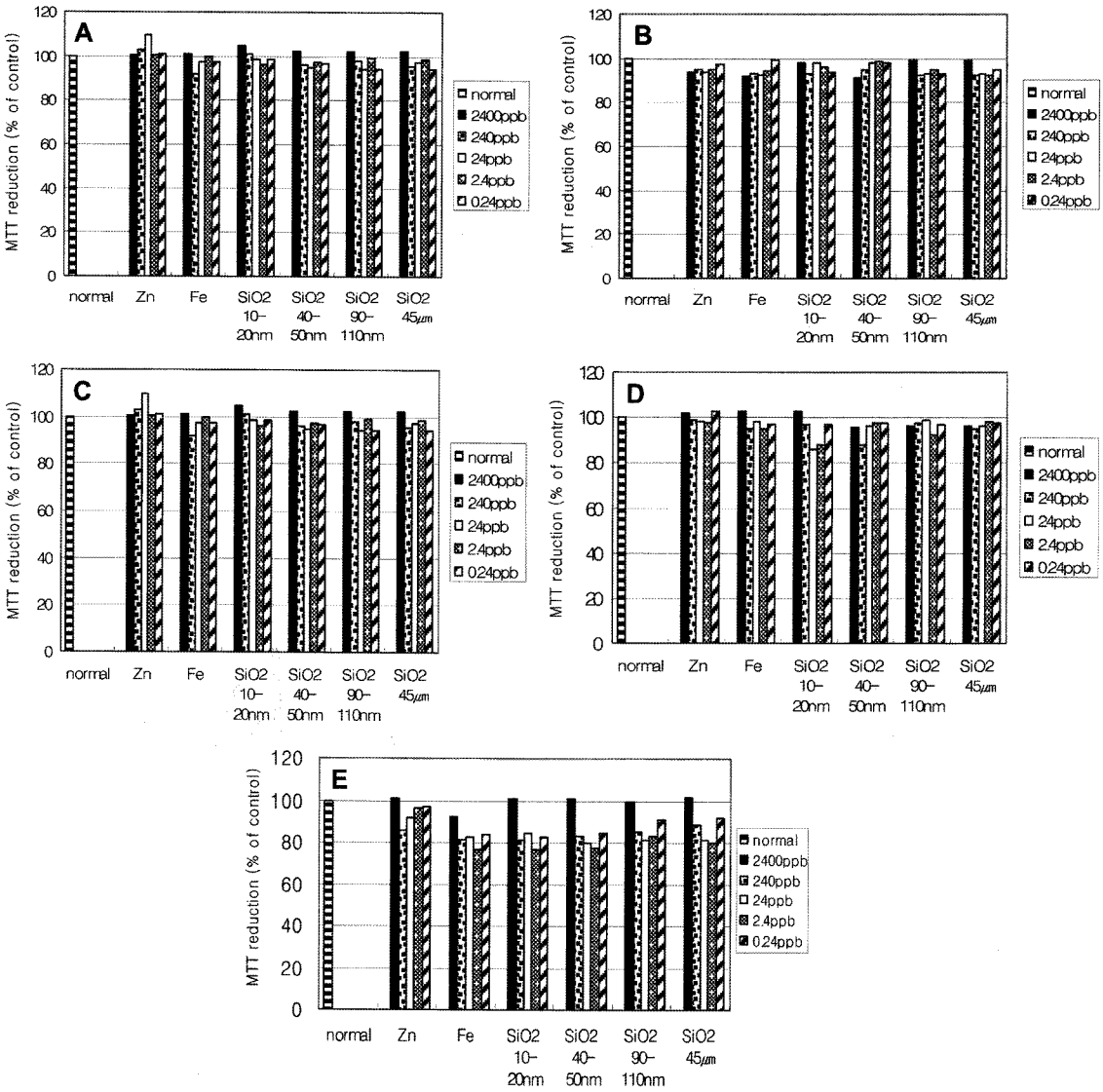

Fig. 1. Cell viability checked with MTT assay. Each cell line was treated with the indicated amount of nanoparticles and the mitochondrial activity measured in 72 h.

A. Liver cell line (Huh-7); **B.** Brain cell line (A-172); **C.** Stomach cell line (MKN-1); **D.** Lung cell line (A-549); **E.** Kidney cell line (HEK293).

sectioned and mounted on a slide glass followed by hematoxylin-eosin staining and observation under light microscopy (Green Cross Lab.).

Mitochondrial Activity

First, the effect of nanoparticles for mitochondrial activity was measured. Mitochondrial dehydrogenase converts MTT tetrazolium to insoluble formazan crystal. Four different concentrations of each nanoparticle were used for the assay (Fig. 1). At lower concentrations of nanoparticles, each cell showed various degrees (0–15%) of mitochondrial activity reduction. However, none showed a concentration dependency, suggesting a nonspecific effect. The three different-sized SiO_2 nanoparticles showed no difference in cytotoxicity. The toxicity was not limited to the presence of nanoparticles since the reduction of mitochondrial activity was the same in the presence of micro-sized particles.

DNA Contents

The DNA contents of a cell reflect its viability. We measured fluorescence from intercalating bisbenzimide H33258 into chromosomal DNA (Fig. 2). Similar to the mitochondrial activity, the amount of DNA was reduced by 0 to 20%. The reduction was readily observed in brain and liver cells, yet stomach and lung cells were less affected. Again, no concentration dependency was shown and the sizes of the nanopartilces did not show any difference. Even a micro-sized SiO_2 particle showed a similar range of cytotoxicity.

Glutathione Production

Glutathione is a naturally occurring antioxidant and produced when a cell is under oxidative stress [9]. When cells were treated with each nanoparticle, 0 to 15% increase in glutathione production was observed. Again,

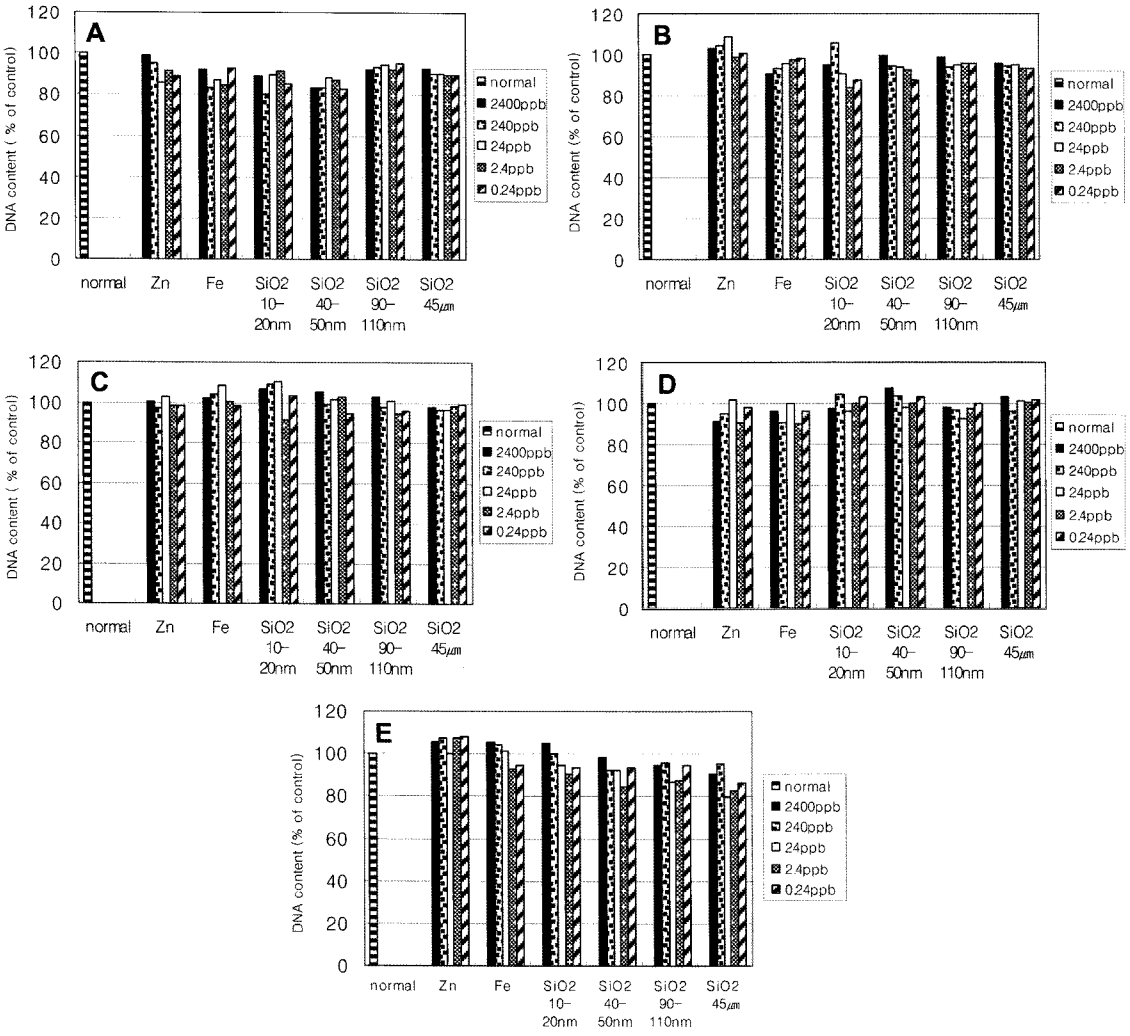

Fig. 2. Cell viability checked with DNA contents. Each cell line was treated with the indicated amount of nanoparticles and the DNA contents measured in 72 h.

A. Liver cell line (Huh-7); **B.** Brain cell line (A-172); **C.** Stomach cell line (MKN-1); **D.** Lung cell line (A-549); **E.** Kidney cell line (HEK293).

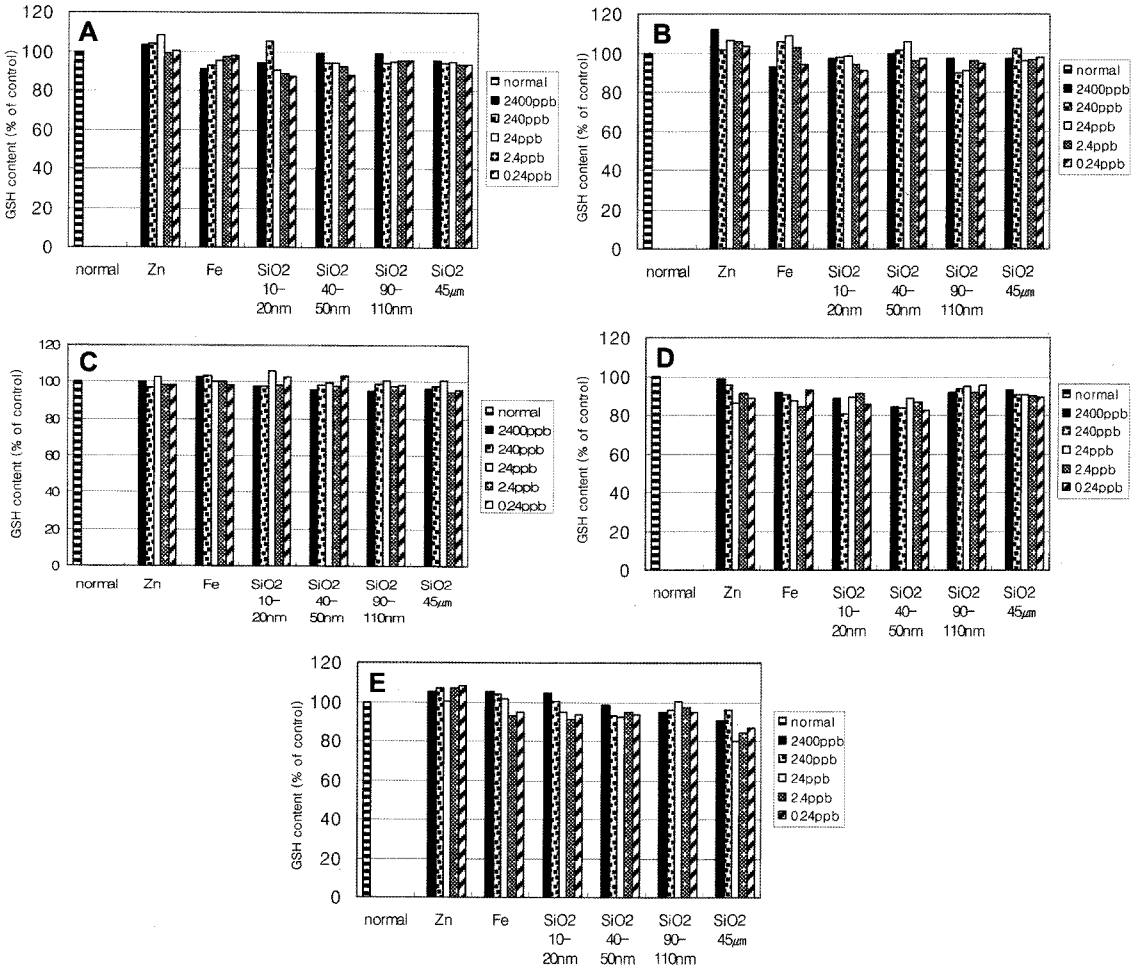

Fig. 3. Oxidative stress observation. Each cell line was treated with the indicated amount of nanoparticles and the glutathione production measured in 72 h.
A. Liver cell line (Huh-7); **B**. Brain cell line (A-172); **C**. Stomach cell line (MKN-1); **D**. Lung cell line (A-549); **E**. Kidney cell line (HEK293).

no concentration dependency was shown and the size of the nanoparticles did not show any difference. Thus, the oxidative stress induced by the presence of nanoparticles was negligible.

Membrane Permeability

We tested whether the presence of nanoparticles alters membrane permeability of a cell, since the plasma membrane is the location where the cell encounters nanoparticles first. After incubation with nanoparticles, the cells were treated with fluorescein-derivatized dextrans. No presence of fluorescence inside the cell was observed under fluorescence microscopy, suggesting that nanoparticles did not alter the membrane permeability (data not shown).

In Vivo Acute Toxicity

The *in vivo* acute toxicity was measured using mice. The mice were fed with various nanoparticles and also with a micro-sized particle as a control, and then histopathological examinations were performed. For the nano-Zn fed mouse (Fig. 4A), a lymphocytic infiltration was observed from the liver portal tract, subepithelial stroma of stomach, and intestine (b, d, and e, respectively). A nonspecific focal hemorrhage was seen in the heart (a), and a nonspecific medullary congestion was seen in the spleen (c). For the nano-Fe fed mouse (Fig. 4B), a nonspecific focal hemorrhage was observed from the heart (a). A nonspecific medullary congestion was seen in the spleen (c). A nil lesion was found from the stomach (d). For the nano-Si fed mouse (Fig. 4C), a nonspecific focal hemorrhage was seen in the heart and liver (a, and b, respectively). Other organs were not affected. It generally seemed that the toxic effect was not specific. Thus, we investigated whether micro-sized particles caused the same effect (Fig.4 D). In the heart, a nonspecific focal hemorrhage of myocardium was shown (a). A focal hemorrhage was shown from the liver and spleen (b and c, respectively). A nil lesion was shown from the stomach and intestine (d and e, respectively). The mild

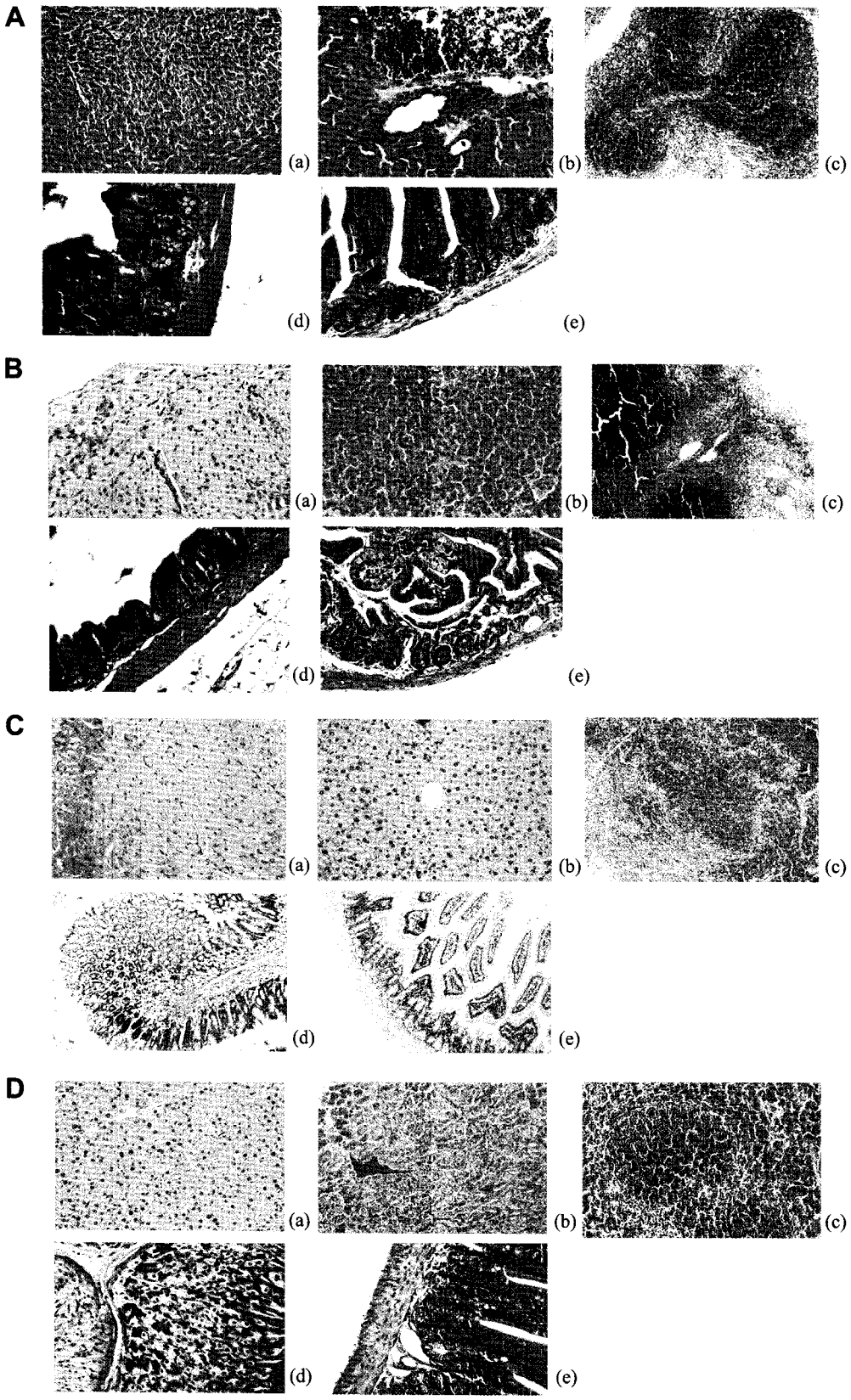

Fig. 4. Hematoxylene-eosin staining of organs from mice fed with indicated nanoparticles (400×).
A. Nano-Zn fed. a. heart; b. liver; c. spleen; d. stomach; e. intestine. **B**. Nano-Fe fed. a. heart; b. liver; c. spleen; d. stomach; e. intestine. **C**. Nano-Si (10–20 nm) fed. a. heart; b. liver; c. spleen; d. stomach; e. intestine. **D**. Micro-Si (45 μm) fed. a. heart; b. liver; c. spleen; d. stomach; e. intestine.

toxicity observed from the treatment of nanoparticles was also seen from the treatment with micro-sized particles.

In conclusion, the cytotoxicity of nanoparticles *in vitro* was low, and it was not dependent on the kind of nanoparticles or on the size. *In vivo*, a low level of toxicity was shown. The toxicity was due to the presence of the inorganic particles themselves, but not to the nanometer size.

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