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Original Article

Acute hepatotoxicity and drug/chemical interaction toxicity of 10-nm silver nanoparticles in mice

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ABSTRACT — Nanomaterials with nanoscale microstructures have new properties in which reactions to stimuli such as heat, light, and voltage differ from those of macroscale materials. For that reason, the development of nanotechnology using nanomaterials has been remarkable, and these technologies have been put to practical use in various fields such as medicine and electronics. Nanomaterials have been researched as new materials with superior properties that have not been seen in the past, but concerns remain about the influence of nanomaterials on living bodies. Silver nanoparticles are materials with excellent optical, electrical, and antibacterial properties. However, few reports have described the influence of silver nanoparticles on the living body and interactions between chemicals such as pharmaceuticals. We therefore investigated the effect of silver nanoparticles on the living body and drug interactions. We administered silver nanoparticles with particle diameters of 10, 50, and 200 nm (SnP10, SnP50, and SnP200, respectively) to mice through the tail vein. As a result, acute liver injury was induced only in the SnP10 group. Furthermore, liver injury was induced by co-administering SnP10 with carbon tetrachloride, streptomycin, or cisplatin. SnP10 appears to induce liver injury through acute and drug interactions.

Key words: Silver nanoparticles, Liver injury, Carbon tetrachloride, Cisplatin, Streptomycin

INTRODUCTION

Recently, nanotechnology has been used in fields such as materials, food, information, and biotechnology as an important basic technology for industry (Layton, 2008; Sekhon, 2010; Chakraborty *et al*., 2011). Nano-scale materials used for nanotechnology have been put to practical use in microelectronics, cosmetics, and sunscreens, and their potential use in drug delivery systems has been considered (Dobson, 2006; Nohynek *et al*., 2008). Nanomaterials exhibit new properties in terms of reactions to heat, light, and voltage stimuli that are not seen in micro-size materials (Wang and Fang, 2013; Chakraborty and Pradeep, 2017). Accordingly, the reduction of particle size from microscale to nanoscale may be beneficial for many industrial and scientific applications. However, nanomaterials may also have toxicities not found in micromaterials, and an understanding of the biological activity and potential toxicity of nanomaterials is essential (Cohen *et al.,* 2013).

Silver is chemically stable and shows high electrical and thermal conductivity and visible light reflectance (You *et al.*, 2012; Reznickova *et al.,* 2015). Silver is used in many products, including chemical instruments, coins, accessories, and medical instruments (Souza *et al.,* 2018). The synthesis of silver nanoparticles has been the subject of extensive research. Silver nanoparticles reportedly offer excellent optical and electrical properties

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and high antimicrobial properties (Iravani *et al.*, 2014; Reznickova *et al.*, 2015). Given these characteristics, silver nanoparticles are being used in an increasingly wide variety of products, such as solar cells, sensors, materials to protect from bacteria, and medical products (You *et al.,* 2012; Zhao *et al.,* 2017). Silver nanoparticles are potentially useful for living organisms due to their antibacterial properties. However, few reports have examined the toxicity of silver nanoparticles in living organisms, and none appear to have investigated latent drug interactions.

As researchers are exploring the safety, pharmacology, and pharmacokinetics of nanoparticles, the field of nanotechnology is expanding. Silica nanoparticles have been shown to cause cytotoxicity, hepatotoxicity, and placental damage (Nishimori *et al.,* 2009b; Yamashita *et al.*, 2011). Another report found that carbon nanotubes can induce pulmonary mesothelioma (Park *et al.*, 2011). However, the pharmacological effects resulting from the interactions between nanoparticles and drugs are not generally known. In this study, the toxicity of silver particles of 10 nm, 50 nm, and 200 nm in diameter (SnP10, SnP50, and SnP200, respectively) was investigated in mice to clarify their safety in mammals. In addition, we examined the effects of these nanoparticles on the toxicity of chemicals such as carbon tetrachloride (a well-known hepatotoxin) (Weber *et al.,* 2003), cisplatin (a widely used antitumor agent) (Ozols and Young, 1991; Witjes, 1997), and streptomycin (a common antibiotic) (Goralczyk *et al.,* 2017).

MATERIALS AND METHODS

Materials

Silver particles with diameters of 10, 50, or 200 nm were obtained from NANOCOMPOSIX, Inc. (San Diego, CA). The size distribution of silver particles was analyzed using a Zetasizer (Sysmex Co., Kobe, Japan) and TEM JEOL JEM-1011 transmission electron microscope: mean diameters were 12.1 ± 3.3 nm, 48.0 ± 18.2 nm, and 239.7 ± 103.2 nm (Fig. 1A-C). Aqueous suspensions of 1 mg/mL were prepared. These suspensions were thoroughly dispersed using sonication before use and were diluted with water. Whether ionized silver was present in the suspension of silver nanoparticles was examined by ICP-MS, but no ionized silver was detected. Identical volumes of suspension were injected in each experiment. Geometric sizes of particles were characterized using a TEM JEOL JEM-1011 transmission electron microscope. Carbon tetrachloride (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in olive oil. Cisplatin (Wako Pure Chemical Industries) and streptomycin (Nakalai Tesque Inc., Kyoto, Japan) were dissolved in saline and stored at -20ºC until use. All reagents were research-grade.

Animals

Eight-week-old BALB/c male mice were purchased from Funabashi Farm Co., Ltd. (Chiba, Japan). The average weight of the animals was 24.8 ± 1.1 g. Mice were maintained in a controlled environment (temperature: 23 ± 1.5°C; 12-hr light/dark cycle) with free access to standard rodent chow and water. The mice were given 1 week to acclimate before the experiments were conducted. All experimental protocols conformed to the ethical guidelines of Teikyo Heisei University Graduate School of Pharmaceutical Sciences, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Sciences.

Preparation and administration of test items

The test item SnP10 was suspended in distilled water for injection and administered intravenously to mice at doses of 0.1, 0.5, 1.0, 2.0, and 4.0 mg/kg body weight. Blood was recovered 24 hr after administration of the test item.

Dose dependency of SnP10

SnP10 was administered to mice intravenously at a dose of 4 mg/kg body weight, blood was recovered 3, 6, 12, 24, 48, and 72 hr after administration, and then the mice were sacrificed (n = 4 for each group).

Drug interactions of silver nanoparticles

SnP10, SnP50, or SnP200 was suspended in water for injection and administered intravenously at a dose of 1 mg/kg body weight. Simultaneously, carbon tetrachloride (0.01 mL/kg), streptomycin (500 mg/kg), or cisplatin (100 μmol/kg) was administered intraperitoneally. Blood was recovered 24 hr after this co-administration. These doses of cisplatin, streptomycin, and carbon tetrachloride were previously determined experimentally to not induce toxicity.

Biochemical analyses

Serum ALT and AST were measured using commercially available kits (Wako Pure Chemical Industries) according to the manufacturer's protocols. Briefly, collected serum (10 mL) was combined with 1 mL of color A reagent (including urease) and incubated at 37°C for 15 min. Following the addition of 1 mL of color B reagent, the sample was incubated at 37°C for 10 min. Absorbance was measured at a wavelength of 570 nm.

TNF-α and IL-6 were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, USA). All analyses were performed in strict accordance with the instructions from the manufacturer.

Histologic analyses

At 24 hr after dose administration, animals were sacrificed, and the livers were removed and fixed with 4% paraformaldehyde. Following processing and sectioning, thin tissue sections were stained with hematoxylin and eosin for histologic observation.

Ultrastructure of liver tissue

The mice were sacrificed on 24 hr after injection of nanoparticles. Mice were fixed in 4% paraformaldehyde in PBS solution by perfusion fixation. The liver tissue samples were collected and fixed in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), post osmicated, and dehydrated with graded alcohol. After immersion in propylene oxide, the specimens were embedded in Epon 812. Ultrathin sections were prepared and collected on electron microscopic grids and examined with a transmission electron microscope (JEM-1011; JEOL).

Statistical analyses

Statistical analyses were performed using Microsoft Excel with the Statcel add-in (EMS Publication Co., Ltd., Saitama, Japan). All data are presented as means ± SEMs. The significance of differences between the control and experimental groups was assessed using Dunnett's test. A value of $P < 0.05$ was considered indicative of statistical significance.

RESULTS AND DISCUSSION

We first measured the particle sizes of silver nanoparticles using a Zetasizer (Sysmex Co.), then observed using transmission electron microscopy (Fig. 1A-C). Mean diameters of the SnP10, SnP50, and SnP200 nanoparticles were 12.1 ± 3.3, 48.0 ± 18.2, and 239.7 ± 103.2 nm, respectively. Furthermore, silver nanoparticles aggregate when measured by electron microscopy, but did not aggregate when administered. In addition, we measured silver ion concentrations by ICP-MS but could not detect silver ions (data not shown).

Initially, we examined whether SnP was hepatotoxic by administering maximum dose of 4 mg/kg to mice from the tail vein. Results are shown in Fig. 2A. ALT and AST levels in the SnP50 or SnP200 groups were almost the same as in the control, and no liver injury was observed. In the SnP10 administration group, the ALT level was 453 ± 200 K.U., and the AST level was 770 ± 220 K.U., resulting in acute hepatic injury. Furthermore, from hematokin-eosin stained images of the liver, the SnP10 administration group showed no nuclei in hepatocytes, and cytotoxicity was observed (Fig. 2B). These findings indicate that SnP10 induced acute hepatic injury.

Next, the dose dependence of SnP10 with liver injury and the time course of liver injury were examined. The results are shown in Fig. 3A and B. Liver injury with SnP10 was induced from a dosage of ≥ 2 mg/kg. No liver injury was seen in SnP10 for doses ≤ 1 mg/kg (Fig. 3A). In the time course results for liver injury with SnP10 at a dose of 4 mg/kg (Fig. 3B), both ALT and AST showed maximum values at 24 hr after administration and returned to normal values after 72 hr.

We have reported that liver injury is induced by co-administering silica nanoparticles, nanoclay, or polystyrene nanoparticles with pharmaceuticals or chemicals (Nishimori *et al.*, 2009a). Therefore, carbon tetrachloride as a hepatotoxin, and streptomycin or cisplatin as agents with hepatotoxic adverse effects were co-administered with silver nanoparticles and investigated. The results are shown in Fig. 4. Co-administration of SnP10 and carbon tetrachloride resulted in elevation of both ALT and AST

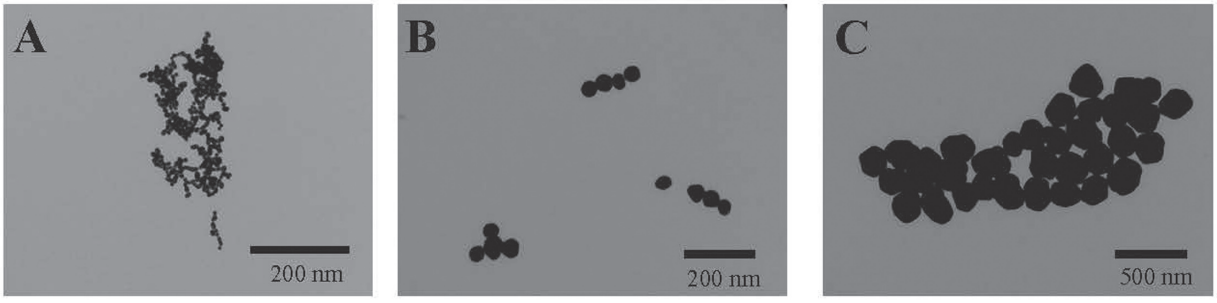

Fig. 1. Ultrastructure of platinum nanoparticles. Electron micrographs of SnP10 (A), SnP50 (C) and SnP200 (C) nanoparticles.

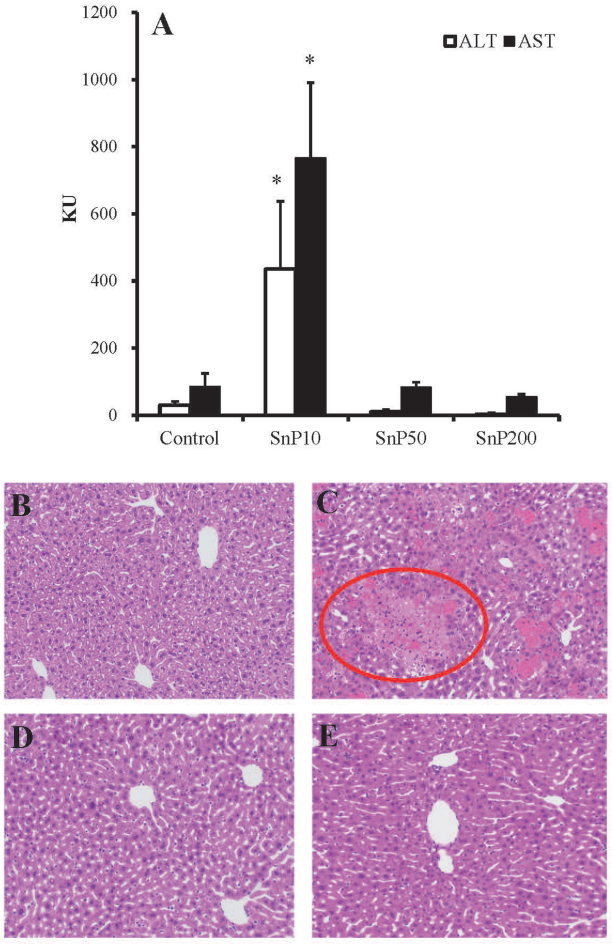

Fig. 2. Comparison of acute liver toxicity of silver nanoparticles. Serum ALT and AST (A) at 24 hr were measured using a commercially available kit, as described in the MATERIALS AND METHODS. Histological analysis of tissues in silver nanoparticle-treated mice. At 24 hr after administration, livers were collected from Control (B), SnP10 (C), SnP50 (D), and SnP200 (E) groups and fixed with 4% paraformaldehyde. Tissue sections were stained with hematoxylin and eosin and observed under a microscope. Data are representative of at least four mice. Blood was recovered at 24 hr after injection. The arrow shows an area of injured liver. Data represent mean ± SEM (n = 4). *Significant difference compared with the control group ($P < 0.05$)

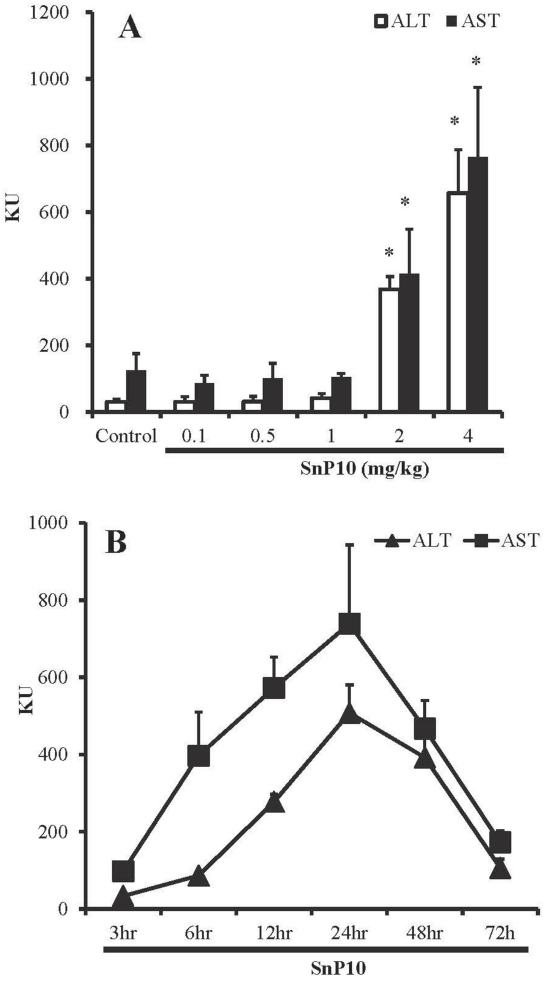

Fig. 3. Dose dependency and time course of SnP10-induced liver injury. (A) Dose dependency of SnP10-induced liver injury. Serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercially available kits (see 'Biochemical analyses' section) 24 hr after administration of SnP10 at the indicated doses. (B) Time course of SnP10-induced liver injury. Mice were intravenously injected with SnP10 at a dose of 4 mg/kg. Blood was recovered at 3, 6, 12, 24, 48, and 72 hr after injection. Serum ALT and AST were measured using commercially available kits, as described in the 'Biochemical analyses' section. Data are given as mean ± standard error of the mean; n = 4).

and hepatic injury was induced (Fig. 4A, B). In addition, co-administration of SnP10 and streptomycin (SM) increased both ALT and AST and induced hepatic injury (Fig. 4C, D). With co-administration of carbon tetrachloride or SM with SnP50 or SnP200, ALT and AST were almost equivalent to levels in controls, and no hepatic injury was induced (Fig. 4A-D). Furthermore, for co-administration of cisplatin and silver nanoparticles, all mice co-administered SnP10 and cisplatin died. Co-administration of SnP50 and cisplatin increased both ALT and AST,

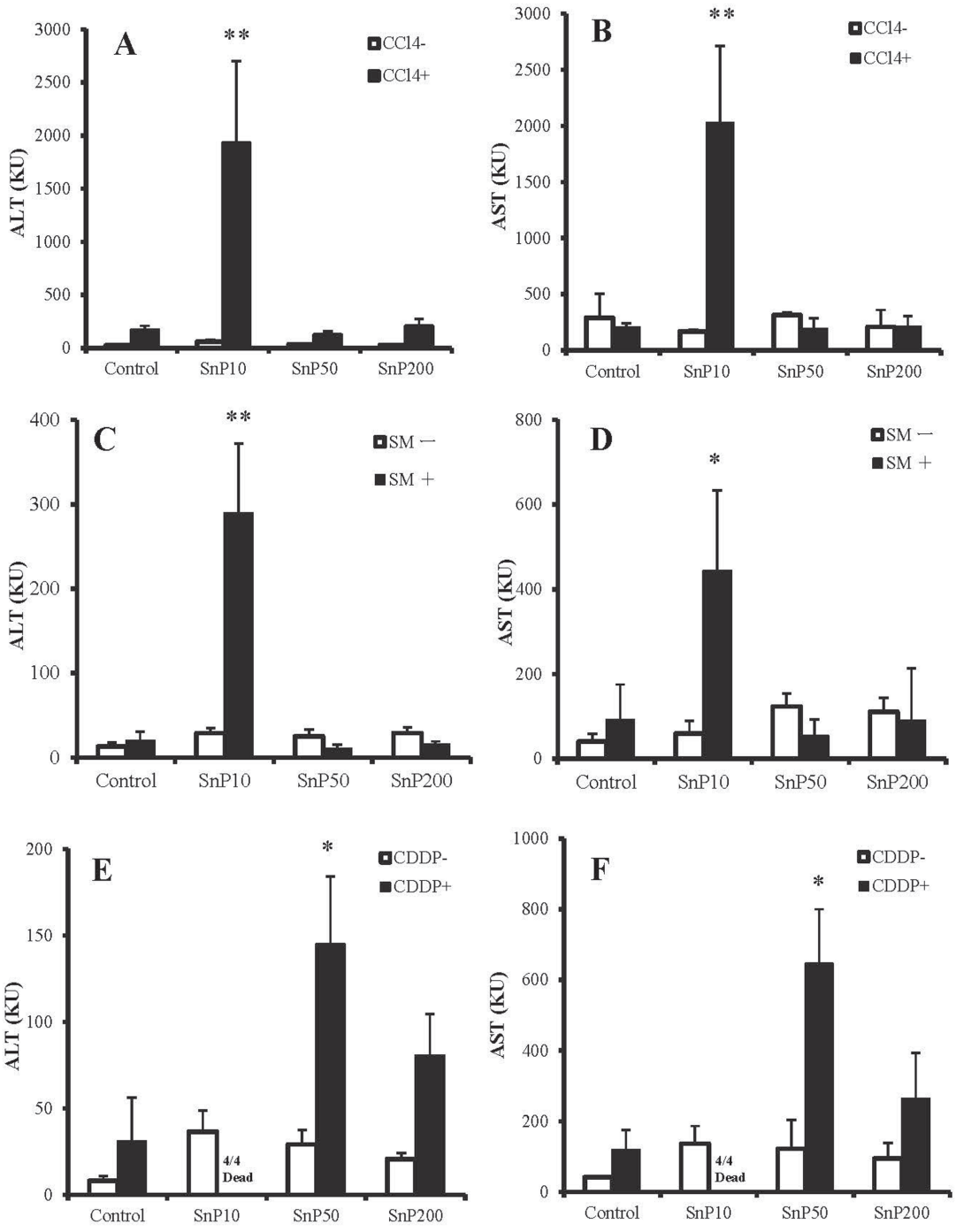

Fig. 4. Effect of co-administration of silver nanoparticles on carbon tetrachloride-, streptomycin- or cisplatin-induced toxicity. Co-administration of SnP and carbon tetrachloride at 0.01 mL/kg: (A) ALT, (B) AST. Co-administration of SnP and streptomycin (SM) at 500 mg/kg: (C) ALT, (D) AST. Co-administration of SnP and cisplatin (CDDP) at 100 μmol/kg: (E) ALT, (F) AST. Mice were injected intraperitoneally with carbon tetrachloride, streptomycin, or cisplatin together with intravenous injection of vehicle or silver particles (1 mg/kg). Blood samples were obtained at 24 hr post-injection. Data are representative of three independent experiments and are presented as the mean ± standard error of the mean (n = 4). Significant difference (*P < 0.05, **P < 0.01) between control- and drug-treated groups.

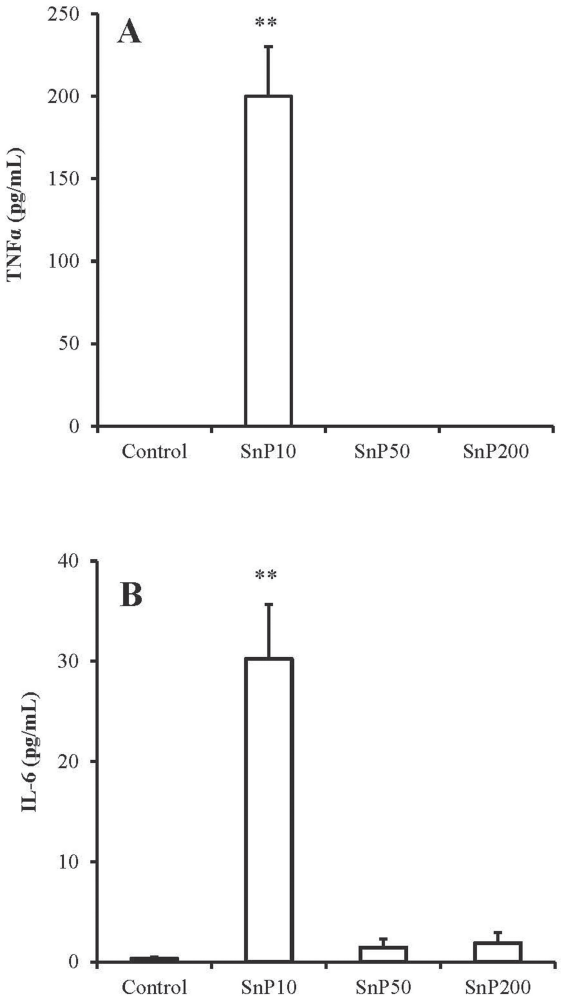

Fig. 5. Serum tumor necrosis factor (TNF)-α (A) and interleukin (IL)-6 (B) levels, as measured by ELISA. Mice received injection of SnP, then cytokine levels were measured 3 hr after administration. Values represent mean ± standard error (SE; n = 4). **P < 0.01 compared with vehicle-treated controls.

and hepatic injury was induced (Fig. 4E, F). These results show that SnP co-administered with pharmaceuticals or chemicals can induce acute liver injury.

Next, to examine the cause of the observed acute liver injury in SnP-treated mice, we measured serum levels of the cytokines TNF-α and IL-6. Serum TNF-α and IL-6 levels were significantly increased 3 hr after SnP10 administration (Fig. 5A, B). Furthermore, we observed the livers of mice administered SnP using transmission electron microscopy (Fig. 6), showing SnP10 invading hepatocytes (Fig. 6B). SnP50 and SnP200 were taken up by Kupffer-cells, but did not invade into hepatocytes (Fig. 6C, D). We found that SnP10 induces acute liver injury by inducing IL-6 and TNF-α and invading hepatocytes.

This study provided the first evidence that acute liver injury can be induced by administering silver particles to mice through the tail vein and that hepatopathy can be induced by co-administration of carbon tetrachloride, cisplatin, or streptomycin with silver nanoparticles.

Acute hepatic injury was induced only in mice administered SnP10 (Fig. 2). Liver injury was not induced with SnP50 or SnP200. In addition, acute hepatic injury was induced when doses of 2 or 4 mg/kg of SnP10 were administered from the mouse tail vein, and SnP10-induced liver injury was dose-dependent (Fig. 3). No kidney injury was encountered because BUN did not increase with administration of SnP10 (data not shown). Furthermore, no damage was observed from the lungs of mice to which SnP10 had been administered, or from tissue staining images of the heart (data not shown). From these findings, we considered that liver injury was induced only by administration of SnP10. Previously, we reported that silica with a particle size of 70 nm accumulated in the liver and induced acute liver injury (Nishimori *et al.*, 2009b). Guo *et al*. also reported that injuries to the liver, lungs, and kidneys were observed 7 days after administration of silver nanoparticles to mice (Guo *et al.,* 2016). We found that silver nanoparticles induced acute liver injury according to nanoparticle size (Fig. 1). Recordati *et al*. reported that silver nanoparticles with a diameter of 10 nm induce hepatic injury, consistent with our results (Recordati *et al.*, 2016). Our findings suggest that silver nanoparticles induce acute hepatic injury at particle sizes of 50 nm or less. We are currently investigating the hepatotoxicity of silver nanoparticles with particle diameters ranging from 10 nm to ≤ 50 nm.

We also investigated the combined effects of various chemicals on SnP10-induced toxicity and found that carbon tetrachloride, streptomycin, and cisplatin show synergistic toxic effects with SnP10. Previously, in our study, silica nanoparticles with a particle size of ≤ 100 nm induced liver damage through interactions with carbon tetrachloride, streptomycin, and cisplatin (Nishimori *et al.*, 2009a). In addition, nanoclay and nano-platinum particles induced liver and kidney injury by interactions with cisplatin (Isoda *et al.*, 2017a; Isoda *et al.*, 2017b). In this study, silver nanoparticles induced only liver injury due to interactions with drugs. Liver injury due to drug interactions was thought to be induced by silver nanoparticles invading intracellularly, based on the electron microscopic images in which SnP10 was seen in hepatocytes

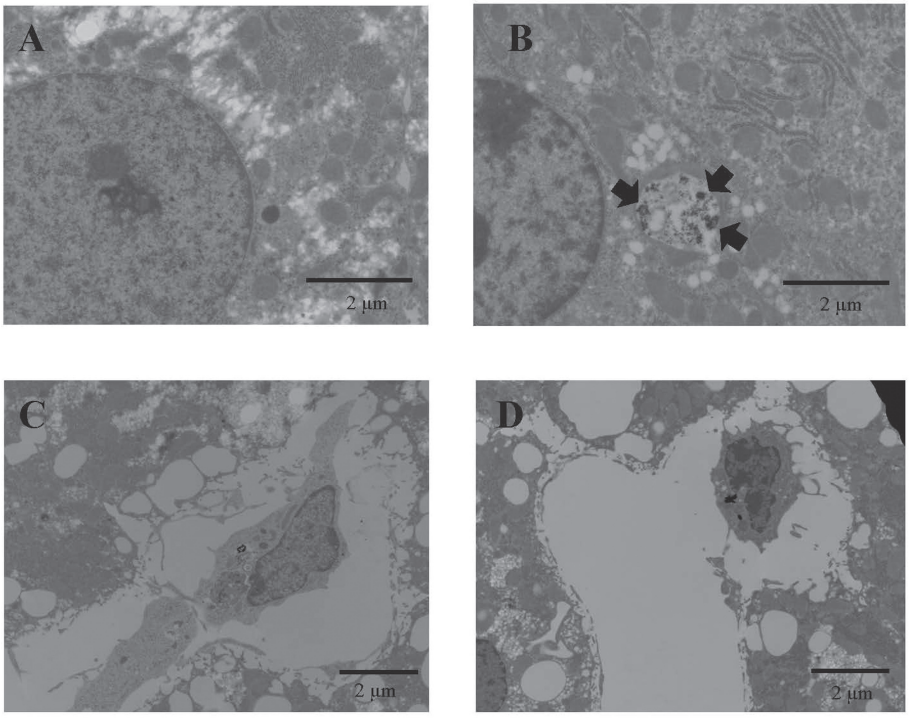

Fig. 6. Ultrastructure of liver tissue treated by silver nanoparticles. Ultrastructure of liver tissue in 10-nm silver nanoparticle-treated mice. At 24 hr after administration, liver tissue was collected from vehicle (A), SnP10 (B), SnP50 (C), and SnP200 (D) groups and fixed with 2.5% glutaraldehyde. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under electron microscopy. Black arrow indicates a silver nanoparticle (SnP10).

(Fig. 6). Further studies are needed to elucidate the mechanisms underlying interactions between silver nanoparticles and drugs in cells.

TNF-α and IL-6 concentrations in the blood were greatly increased with the administration of SnP10 (Fig. 5). TNF-α and IL-6 are cytokines induced during acute liver injury (Liu *et al.,* 2017). In addition, TNF-α and IL-6 are reportedly involved in immune responses and induce hepatic injury (Tacke *et al.,* 2009). We found SnP10 invaded into hepatocytes from transmission electron microscopic images (Fig. 6). These results suggest that acute liver injury caused by SnP10 resulted from direct injury by particles and from immune reactions altering signal transduction. However, the detailed intracellular molecular mechanisms underlying liver injury by SnP10 are unknown. In the future, additional investigations are needed to elucidate the mechanisms underlying the liver injury caused by SnP10.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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