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Research paper

# Silica nanoparticles as hepatotoxicants

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ABSTRACT

Nano-size materials are increasingly used in cosmetics, diagnosis, imaging and drug delivery, but the toxicity of the nano-size materials has never been fully investigated. Here, we investigated the relationship between particle size and toxicity using silica particles with diameters of 70, 300 and 1000 nm (SP70, SP300, and SP1000) as a model material. To evaluate acute toxicity, we first performed histological analysis of liver, spleen, kidney and lung by intravenous administration of silica particles. SP70-induced liver injury at 30 mg/kg body weight, while SP300 or 1000 had no effect even at 100 mg/kg. Administration of SP70 dose-dependently increased serum markers of liver injury, serum aminotransferase and inflammatory cytokines. Repeated administration of SP70 twice a week for 4 weeks, even at 10 mg/kg, caused hepatic fibrosis. Taken together, nano-size materials may be hepatotoxic, and these findings will be useful for future development in nanotechnology-based drug delivery system.



## 1. Introduction

The recent development of technology for reducing material size has provided innovative nanomaterials. Nanomaterials are engineered structures with at least one dimension of 100 nm or less, and have unique physicochemical properties with regard to size, chemical composition, surface structure, solubility, shape and aggregation. Nanomaterials have been widely used in microelectronics, catalysts, ultra-sensitive molecular sensing and imaging probes, pharmaceutical agents and cosmetics. Thus, the development of reduced particle size from the macro to the nanoscale provides benefits to a range of industrial and scientific fields. However, materials that are inert in bulk form may be toxic in nano-size forms, and it is thus essential to understand the biological activities and potential toxicity of nanomaterials [1–3].

The influence of inhalation of nanomaterials on human health has been widely investigated. Occupational exposure to quartz, mineral dust particles and asbestos induce inflammation, fibrosis and cytotoxicity in the lung [3]. In animal models, inhaled nanoparticles do not locally remain in the lung, and pass into blood flow, resulting in distribution to distant organs, such as the liver, kidney, brain and heart [4–7]. Moreover, biomedical applications for diagnosis and therapeutic purposes will require intravenous, subcutaneous or intramuscular administration [8–10]. Thus, it is necessary to confirm the influence of nanomaterials in systemic flow on various organs.

Silica nanoparticles have been applied to diagnosis and drug delivery [4,11], and intraperitoneal administration of silica nanoparticles results in the biodistribution of the nanoparticles to diverse organs, such as the liver, kidney, spleen and lung [4]. Both micro- and nano-size silica particles are also commercially available. In the present study, we investigated the influence of nanomaterials on major organs, such as the liver, kidney, spleen and lung using silica particles as a model material. When silica particles with a diameter of 70, 300 or 1000 nm were intravenously injected, only the 70-nm particles led to acute and chronic liver injury.

## 2. Materials and methods

### 2.1. Materials

Silica nanoparticles with a diameter of 70, 300 or 1000 nm were purchased from Micromod Partikeltechnologie GmbH (Rostock,

*Abbreviations:* SP70, 70 nm silica particles; SP300, 300 nm silica particles; SP1000, 1000 nm silica particles; ALT, aminotransferase; BUN, blood urea nitrogen; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; $GdCl_3$, gadolinium chloride; CPA, cyclophosphamide; LSEC, liver sinusoidal endothelial cells; MARCO, macrophage receptor with collagenous structure.

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Germany). The average size of the silica particles was determined to be 75.7, 311 and 830 nm by Zetasizer (Sysmex Co., Kobe, Japan). The particles were spherical and nonporous. The particles were stocked at 25 mg/ml (70 nm) and 50 mg/ml (300 and 1000 nm) in aqueous suspension. The stock solutions were suspended using vortex mixer for 5 min before use. The resultant solutions did not show aggregation of the particles by electron microscopy analysis. Reagents used in this study were of research grade.

### 2.2. Animals

BALB/c male mice (8 wk) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/12-h dark cycle. Mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Mice were intravenously injected with the silica particles at 10–100 mg/kg body weight. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

### 2.3. Histological analysis

The liver, kidney, spleen and lung were removed and fixed with 4% paraformaldehyde. After sectioning, thin tissue sections of tissues were stained with hematoxylin and eosin for histological observation. Liver sections were stained with Azan-Mallory for observation of liver fibrosis.

### 2.4. Biochemical assay

Serum alanine aminotransferase (ALT) levels and blood urea nitrogen (BUN) were measured using a commercially available Transaminase-CII kit and Blood Urea Nitrogen-B Test Wako (WAKO Pure Chemical, Osaka, Japan), respectively. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured with an ELISA kit (BioSource International, CA, USA).

### 2.5. Gadolinium chloride assay

For Kupffer cell blockage of phagocytosis and partial depletion in the liver, mice were injected intravenously with gadolinium chloride ($GdCl_3$) at 10 mg/kg body weight at 30 and 6 h prior to intravenous administration of nanoparticles [12,13]. Blood was then recovered 24 h after injection of nanoparticles for ALT assay.

### 2.6. Cyclophosphamide assay

Disruption of liver sinusoidal endothelial cells was carried out by intraperitoneal injection of 300 mg/kg body weight cyclophosphamide (CPA) at 24 h prior to administration of nanoparticles [14,15]. Blood was recovered at 24 h after injection of nanoparticles for ALT assay.

### 2.7. Hepatic hydroxyproline content

Hepatic hydroxyproline content was assayed by Kivirikko's method, with some modification [16]. Briefly, liver tissue was hydrolyzed in 6 M HCl at 110 °C for 24 h in a glass tube. After centrifugation, the resultant supernatant was neutralized with 8 N KOH, and 2 g of KCl and 1 ml of 0.5 M borate buffer were then added, followed by incubation for 15 min at room temperature and further incubation for 15 min at 0 °C. Chloramine-T solution was then prepared and added. After additional incubation for 1 h at 0 °C, 2 ml of 3.6 M sodium thiosulfate was added, followed by incubation at 120 °C for 30 min. Next, 3 ml of toluene was added with incubation for a further 20 min at room temperature. After centrifugation, 2 ml of the resultant supernatant was added to Ehrlich's reagent, followed by incubation for 30 min at room temperature. Subsequently, the absorbance was measured at 560 nm.

### 2.8. Statistical analysis

Statistical analysis was performed by two-way ANOVA, followed by Student's *t*-test. The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. Liver injury by 70-nm silica nanoparticles

We initially investigated the acute toxicity of silica particles with diameters of 70 (SP70), 300 (SP300) or 1000 nm (SP1000) at maximal dose of 100 mg/kg. Intravenous injection of SP70 at 50 and 100 mg/kg was often lethal, but mice injected with SP300 and SP1000 survived. Fig. 1 shows hematoxylin–eosin staining of the liver, spleen, lung and kidney in silica particle-injected mice. We found no toxicity in any of these organs in SP300 or SP1000-injected mice at 100 mg/kg, and we found no abnormalities in the spleen, kidney and lung in SP70-injected mice at 30 mg/kg (Fig. 1A–D). However, degenerative necrosis of hepatocytes in the liver was observed in SP70-injected mice, thus suggesting that SP70 is toxic to the liver (Fig. 1A).

Next, in order to confirm the hepatotoxicity of SP70, we examined serum ALT activity, a biochemical marker of liver injury. Consistent with the histological data, injection of SP70 elevated serum ALT levels 35-fold over control values at 30 mg/kg, but injection of SP300 or SP1000 had no effect even at 100 mg/kg (Fig. 2A). Elevation of blood urea nitrogen, a biochemical marker of kidney injury, was not observed (Fig. 2B). Serum levels of inflammatory cytokine IL-6 and TNF-α were markedly elevated to 1124 and 80 pg/ml, respectively, in SP70-treated mice at 3 h (Fig. 2B and C). Slight elevation of serum IL-6 levels was observed in SP300- and SP1000-injected mice (28 and 32 pg/ml, respectively), while no elevation of TNF-α was observed. The IL-6 levels seen in SP300- or SP1000-treated mice were insufficient for liver injury. To investigate dose dependency of SP70-induced liver injury, we also investigated serum ALT and inflammatory cytokine levels at 12 h after SP70 administration. ALT, IL-6 and TNF-α levels were elevated in a dose-dependent manner after SP70 injection, and significant increases were observed with doses as low as 20 mg/kg (Fig. 3A–C). Taken together, these data suggest that 70-nm silica particles are toxic to the liver.

### 3.2. Involvement of Kupffer cells in SP70-induced liver injury

Kupffer cells are large liver macrophages, and are localized within the liver sinusoidal cells. Kupffer cells play a role in defense against various particles and substances entering the liver through the portal circulation [17]. Indeed, Kupffer cells clear virus particles from the bloodstream by phagocytosis [18–20]. The phagocytosis of parasites by Kupffer cells is accompanied by the release of pro-inflammatory cytokines that act as a paracrine signal to neighboring hepatocytes, and induce chemotaxis and aggregation of neutrophils. $GdCl_3$ inhibits phagocytosis by Kupffer cells and transiently eliminates Kupffer cells [12], and $GdCl_3$ has thus been widely used to investigate the roles of Kupffer cells in the liver [21,22]. To investigate the involvement of Kupffer cells in particle-induced liver injury, we evaluated the effects of $GdCl_3$ on nanoparticle-induced liver injury. As shown in Fig. 4A, pre-injection of $GdCl_3$ prior to injection of SP70 elevated serum ALT levels 5.5-fold

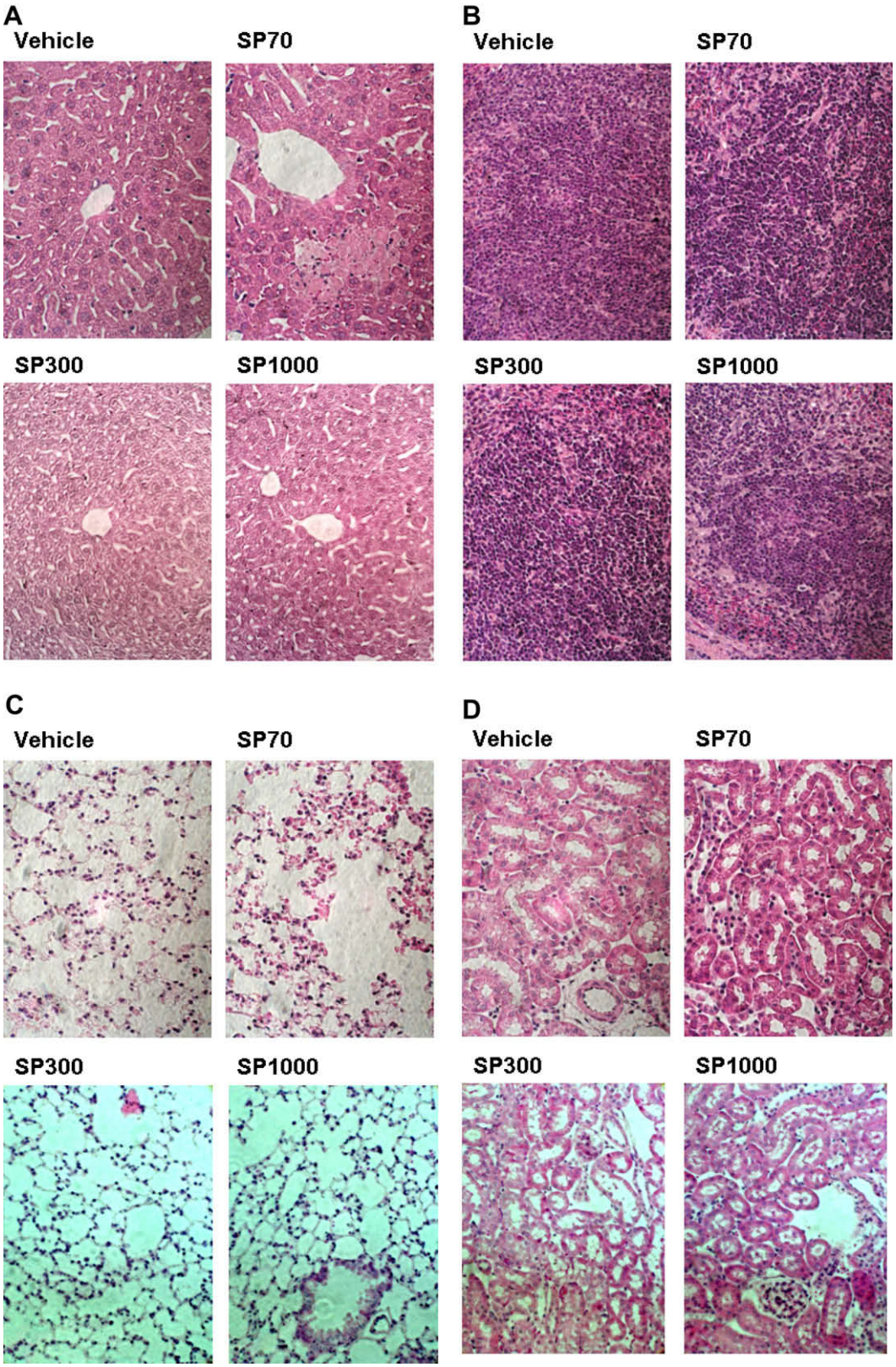


**Fig. 1.** Histological analysis of tissues in silica particle-treated mice. Silica particles with diameters of 70 (SP70), 300 (SP300) or 1000 nm (SP1000) were intravenously administered to mice at 30, 100 and 100 mg/kg, respectively. At 24 h after administration, tissues of liver (A), spleen (B), lung (C) and kidney (D) were collected, 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.

in the SP70-injected group. In contrast, pre-injection of $GdCl_3$ did not affect ALT levels in the SP300- or SP1000-administered group. Taken together, these results indicate that phagocytosis of SP70 by Kupffer cells may attenuate liver injury, but the release of proinflammatory cytokines from Kupffer cells is not associated with SP70-induced liver injury.

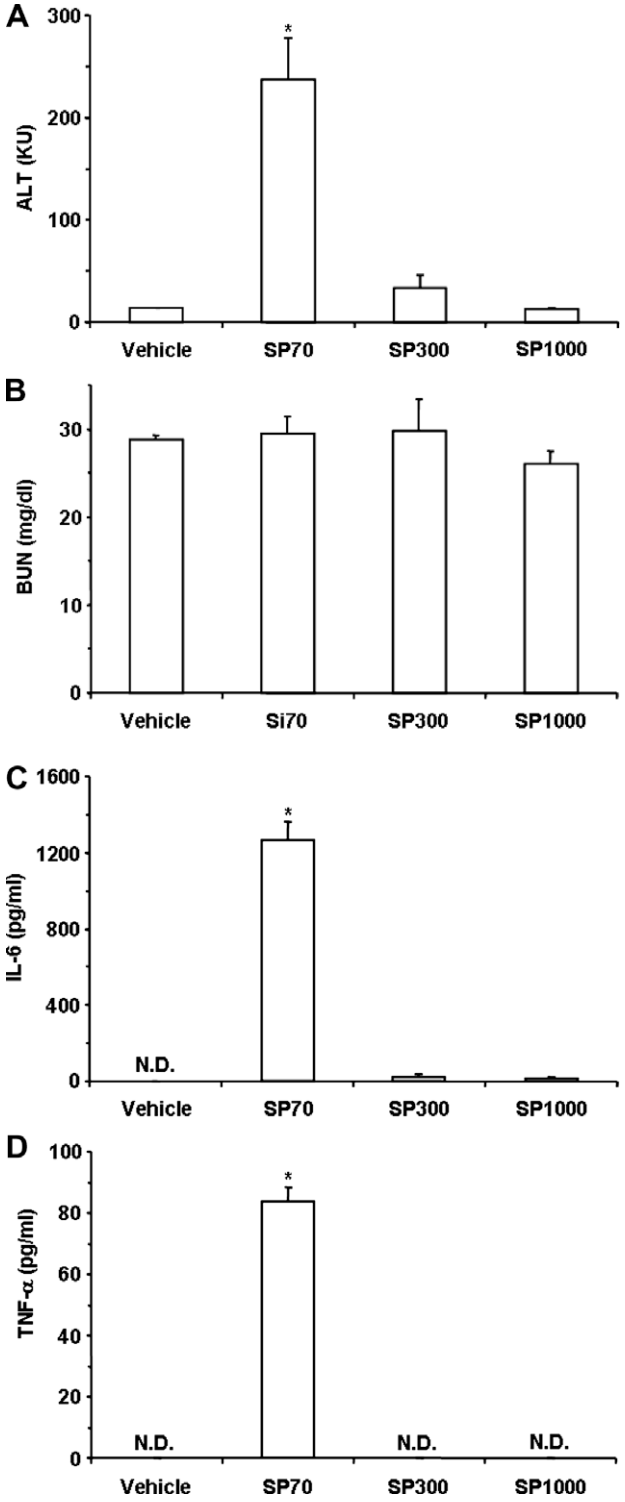


**Fig. 2.** Biochemical analyses of liver injury in silica particle-injected mice. SP70, SP300 or SP1000 was intravenously injected to mice at 30, 100 or 100 mg/kg, respectively. Blood was recovered at 3 and 24 h of the injection. Serum ALT (A) and BUN (B) at 24 h and IL-6 (C) and TNF-α (D) levels at 3 h were measured using a commercially available kit, as described in Section 2. Data are means ± SEM ($n = 4$). *Significant difference vs. vehicle-treated group ($p < 0.05$).

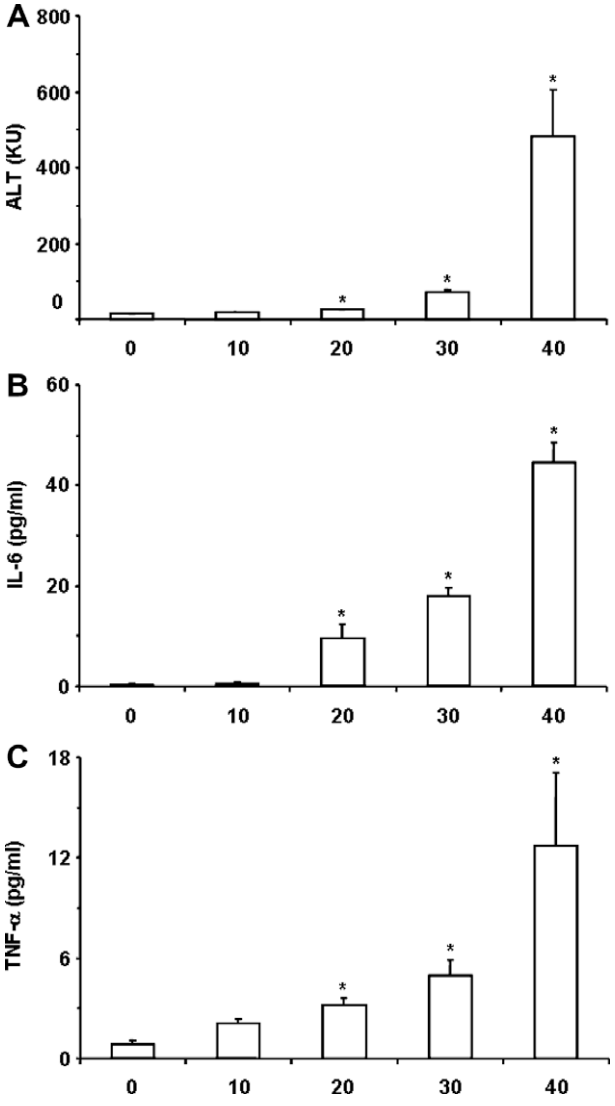


**Fig. 3.** Dose dependency of SP70 on liver injury. SP70 were intravenously administered at the indicated doses, and blood was recovered at 12 h after administration. Serum was used for measurement of ALT (A), IL-6 (B) and TNF-α (C), as described in Section 2. Data are means ± SEM ($n = 4$). *Significant difference compared with the vehicle-treated group ($p < 0.05$).

### 3.3. Involvement of liver sinusoidal endothelial cells in SP70-induced liver injury

Sinusoidal endothelium forms a barrier between the bloodstream and hepatocytes, preventing passage of particles. Liver sinusoidal endothelial cells (LSECs) are perforated by fenestrations, which are pores of approximately 100 nm in diameter. SP70, but not SP300 and SP1000, may pass through LSECs to the hepatocytes, resulting in liver injury. To evaluate this hypothesis, we performed CPA assay. CPA is converted in the liver to toxic metabolites, 4-hydroperoxycyclophosphamide and acrolein, to which endothelial cells are 20-fold more susceptible than hepatocytes [14]. CPA has been shown to disrupt LSECs [14,15]. We thus investigated the effects of CPA on nanoparticle-induced liver injury. As shown in Fig. 4B, pre-injection of CPA did not affect ALT levels in SP300- or SP1000-administered mice, whereas CPA dramatically decreased ALT levels to near control values in SP70-injected mice (from 235 to 29 KU). These data on CPA indicate that LSECs may be directly or indirectly involved in SP70-induced liver injury, but may not be a barrier against SP70.

### 3.4. Chronic toxicity of SP70

Finally, we investigated the effects of SP70 on chronic liver injury. SP70 was injected into mice every 3 days for 4 weeks at 10 or 30 mg/kg. The lower dose (10 mg/kg) did not cause acute liver

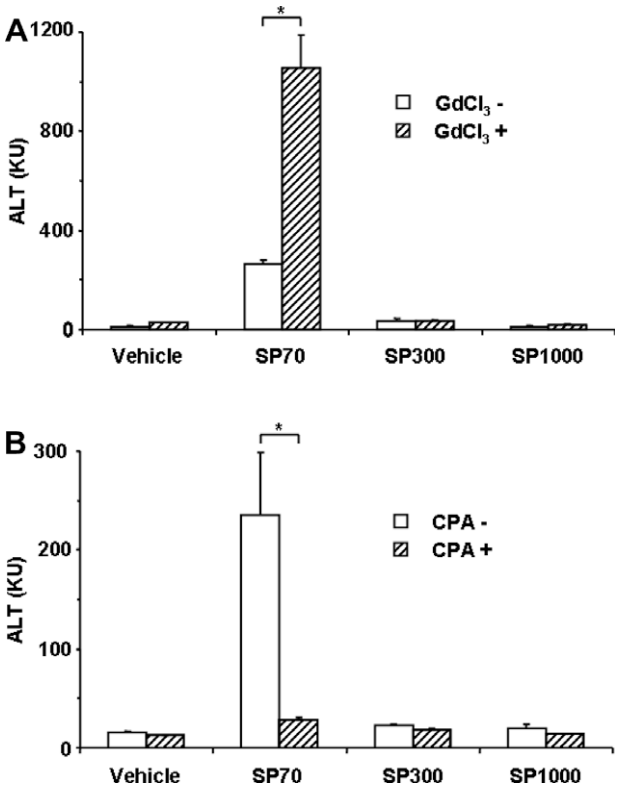


**Fig. 4.** Pharmaceutical analysis of SP70-induced liver injury. (A) $GdCl_3$ assay. Vehicle or $GdCl_3$ (10 mg/kg) was intravenously injected into mice at 30 h or 6 h prior to treatment with silica particles (SP70, 30 mg/kg; SP300, 100 mg/kg; SP1000, 100 mg/kg). At 24 h after particle administration, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM ($n = 4$). *Significant difference between vehicle- and silica particle-treated groups ($p < 0.05$). (B) CPA assay. Vehicle or CPA (300 mg/kg) was intraperitoneally injected to mice at 24 h prior to treatment with silica particles. At 24 h after administration of particles, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM ($n = 4$). *Significant difference between vehicle- and silica particle-treated groups ($p < 0.05$).

failure (Fig. 3A). Histological analysis demonstrated that chronic exposure of SP70-induced denaturation of hepatocytes in a dose-dependent manner (Fig. 5A). Serum ALT levels were also elevated by SP70 administration (Vehicle, 14.3 KU; SP70, 24.8 and 42.1 KU at 10 and 30 mg/kg, respectively) (Fig. 5B). Liver fibrosis is a symptom of chronic liver injury, and thus, we investigated liver fibrosis. Collagen, which is accumulated in the fibrotic liver, was stained with Azan reagent, and blue-stained regions were observed in SP70-treated, but not vehicle-treated, liver sections (Fig. 5C). Elevated hydroxyproline content parallels the extent of fibrosis, and we investigated the hydroxyproline contents in the SP70-treated mouse liver. Injection of SP70 significantly elevated hepatic hydroxyproline contents 1.6- and 3.5-fold over control values, at 10 mg/kg and 30 mg/kg, respectively (Fig. 5D). These data indicate that chronic administration of SP70 causes liver fibrosis, even at doses that are non-toxic in a single injection.

## 4. Discussion

In the present study, we evaluated the acute toxicity of silica particles with a diameter of 70, 300 or 1000 nm, and we found that 70-nm silica particles injure the liver, but not the spleen, lung or kidney. Moreover, chronic administration of 70-nm silica particles caused liver fibrosis, even at doses that were non-toxic in a single injection.

Surface area is a critical factor for toxicity of nano-size particles in the liver. The numbers of particles of SP70, 300 and 1000 are $2.8 \times 10^{12}$, $3.5 \times 10^{10}$ and $9.5 \times 10^{8}$ particles/mg, respectively.

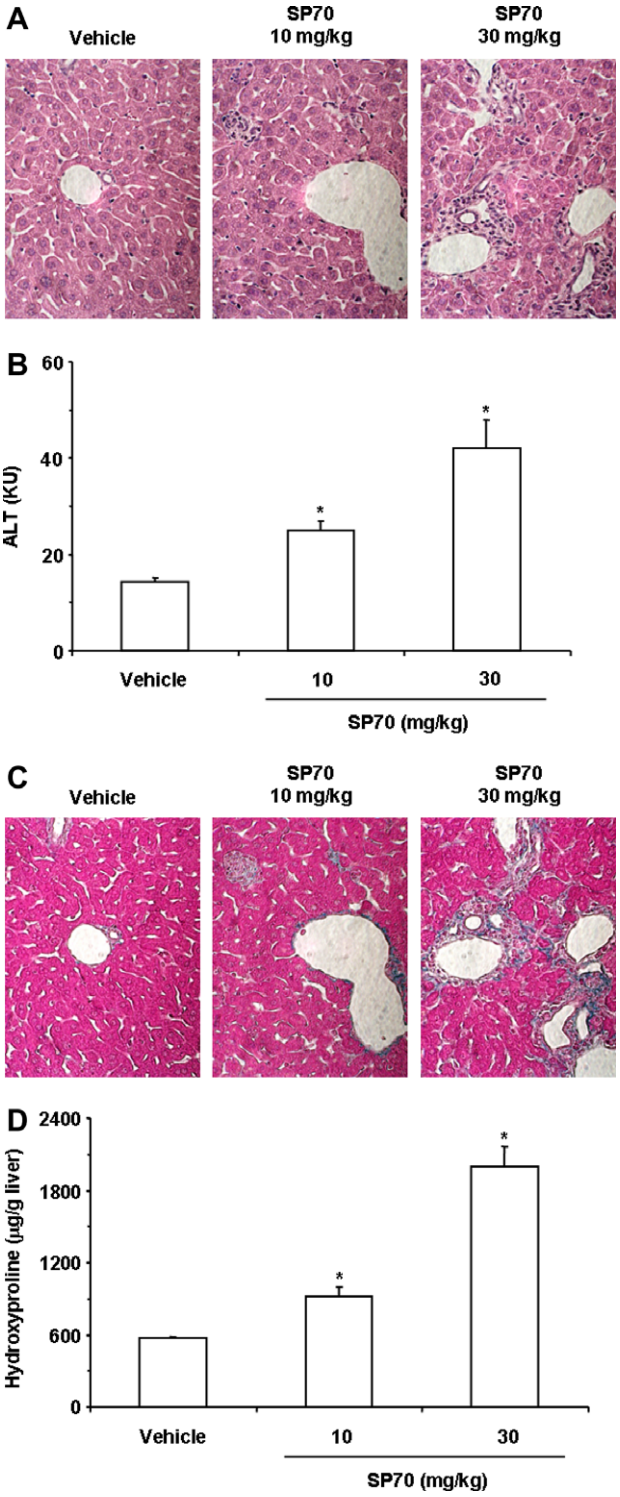


**Fig. 5.** Effect of SP70 on chronic liver injury. Mice were subjected to repeated administration of SP70 (10 or 30 mg/kg) every 3 days for 4 weeks. At 3 days after the last administration, mice were sacrificed. Tissues of livers were fixed with 4% paraformaldehyde, and liver sections were then stained with hematoxylin and eosin (A) or Azan (C). Hydroxyproline levels in the liver were assayed as described in Section 2 (C). Serum samples were used for measurement of ALT (B). (A and C) Data are representative of at least eight mice. (B) Data are means ± SEM ($n = 4$). *Significant difference vs. vehicle-treated group ($p < 0.05$).

The surface area of SP300 at 100 mg/kg, at which SP300 was not toxic, is similar to that of SP70 at 30 mg/kg, at which SP70 was toxic. Difference in the surface area may not affect the different toxicities in the liver between SP70 and 300.

There are highly specialized endothelial cells, LSECs, in the liver, and these separate sinusoidal blood from hepatocytes. Passage of particles through LSECs is the first step for translocation from the bloodstream to hepatocytes. LSECs have fenestrations with a diameter of 100 nm, and the liver injury seen with 70-nm silica particles may be due to the particle size. We investigated the role of LSECs in the particle-induced liver injury using CPA, a disruptor of LSECs [13–15]. Unexpectedly, the disruption of LSEC did not cause SP300- and SP1000-induced liver injury. These results were consistent with the previous report that disruption of LSECs by CPA did not affect the hepatocyte transduction of a lentivirus vector with a diameter of 120–200 nm larger than the fenestrations of LSECs [13]. In contrast, SP70-induced liver injury was dramatically suppressed by disruption of LSECs, and pores in the LSEC may be responsible for the hepatic toxicity of SP70. Spaces, called the space of Disse, exist between LSEC and hepatocytes [23]. Particles entering into these spaces can avoid efflux into the blood flow in the sinusoids of the liver, resulting that this may enhance interaction between the particles and hepatocytes. Thus, the Disse spaces between LSECs and hepatocytes may be responsible for the liver injury caused by SP70.

Resident macrophages in the liver, Kupffer cells play a pivotal role in defense against foreign particles by eliminating such particles via phagocytosis [17]. $GdCl_3$ has been widely used to block phagocytosis by Kupffer cells and to deplete Kupffer cells [12,13,20,21]. Inactivation of Kupffer cells had no effect on SP300 and SP1000 treatment, whereas pre-treatment with $GdCl_3$ led to increased liver injury by SP70. There is no evidence that $GdCl_3$ exerts any direct toxic effects on hepatocytes, LSECs, or on other cells in the liver [24]. Thus, the elevation of SP70 toxicity may be caused by the depletion of Kupffer cells. Depletion of Kupffer cells enhanced the transgene activity of adenovirus vectors with a similar size with SP70 in the liver [22]. Therefore, inhibition of phagocytosis of Kupffer cells may enhance the interaction between SP70 and hepatocytes by increase in SP70 moving into the Disse spaces. Inhalation of silica particles causes lung injury [25], and alveolar macrophages function as a defense against inhaled agents, including viruses and environmental particles, via phagocytosis [26]. Macrophage receptor with collagenous structure (MARCO), CD204 and CD36 are reported to be the receptors for inert particles [26–30]. Uptake of silica particles through MARCO or CD204 induces cytotoxicity in alveolar macrophages, leading to lung fibrosis [30,31]. Alveolar macrophages from BALB/c do not express MARCO and CD204, and silica particles are taken up through CD36 [30]. Thus, uptake of SP70 by Kupffer cells through CD36 might not trigger liver injury. In this study, we found that chronic administration of SP70 caused liver fibrosis, even at 10 mg/kg body weight, at which level acute liver injury was not observed after a single injection. Nano-size particles-induced continuous inflammation in the liver will cause liver fibrosis leading to hepatic cancer.

Further evaluation of relationship between toxicity and variety of sizes, shapes, and chemical modification on the surface of particles is needed, and the future studies based on these data will provide very useful information on future development of drug delivery system using nano-size materials.

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