ORIGINAL ARTICLES

Laboratory of Bio-Functional Molecular Chemistry[1] and Laboratory of Toxicology and Safety Science[2], Graduate School of Pharmaceutical Sciences, Osaka University; Laboratory of Biopharmaceutical Research (Pharmaceutical Proteomics)[3], National Institute of Biomedical Innovation, Osaka, Japan

Hepatotoxicity of silica nanoparticles with a diameter of 100 nm

T. Hasezaki[1], K. Isoda[1], M. Kondoh[1], Y. Tsutsumi[2,3], K. Yagi[1]

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Dr. Kiyohito Yagi, Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan
yagi@phs.osaka-u.ac.jp

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Nanomaterials have potential toxicity that is not found in micromaterials, and it is therefore essential to understand their biological activity and potential toxicity. We focused on silica nanoparticles, since it was previously reported that the intravenous administration of silica nanoparticles with a diameter of 70 nm (SP70) causes hepatic injury. In the present study, we focused on the effects of the particle diameter of silica. We found that silica nanoparticles caused acute liver toxicity at a diameter of 100 nm, and that liver sinusoidal endothelial cells are directly involved in silica nanoparticle-induced liver injury. These findings suggest that the diameter of nanoparticles has great influence on silica nanoparticle-induced liver injury.

1. Introduction

Nanoparticles are generally defined as having diameters of 100 nm or less (Stone et al. 2007; Tsuda et al. 2009). Nanomaterials are used frequently in microelectronics, cosmetics, and semiconductor materials, and research for the development of nanomaterial-based drug delivery systems is promising. As such, there has been a tendency to decrease the grain diameter from the micro to the nano scale in a variety of industrial fields. However, nanosized particles have a potential for toxicity that does not exist for microparticles. It is therefore imperative to understand the biological activity and potential toxicity of nanosized particles (Bystrzejewska-Piotrowska et al. 2009; Warheit et al. 2008).

Silica is the oxide of silicon, and has a large, porous outer structure with a variety of useful characteristics (Kobler and Bein 2008). Silica is commonly used as an industrial material due to its durability and general applicability (Mc Nally et al. 2006). It has been reported that non-crystalloid silica particles at the micrometer scale are completely safe for human exposure (Martin 2007). However, silica nanoparticles are increasingly used as materials in the electronics industry because they serve as a unique substrate (Chung et al. 2009). It is thought that the production of silica nanoparticles will be expanded in the future; however, little is known about their toxicity.

Since nanoparticles are a unique substrate, it follows that their effects on the living body are also unique, and therefore potentially problematic. It is thought that the size and surface area of the particles are important factors in their influence on the living body (Merget et al. 2002). Therefore, decreasing the size of the particles increases their potential influence on living things.

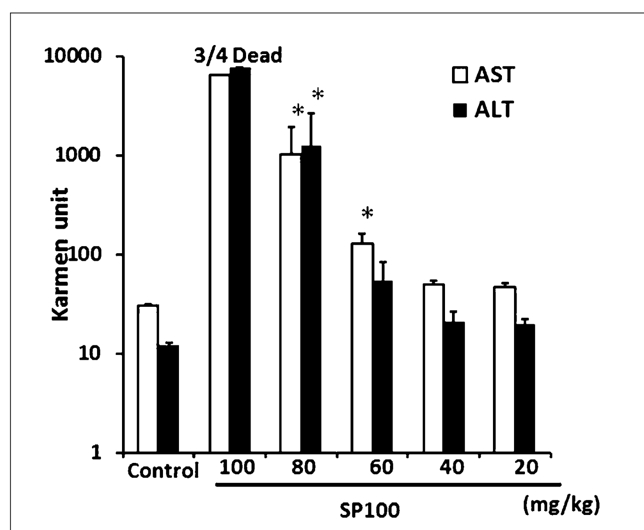
Abbreviations: SP70 70 nm silica particles; SP100 100 nm silica particles; SP300 300 nm silica particles; CDDP cisplatin; PQ paraquat; ALT alanine aminotransferase; AST aspartate aminotransferase; BUN blood urea nitrogen.

We previously reported that silica nanoparticles with a diameter of 70 nm cause liver injury, whereas silica nanoparticles of a diameter of 300 nm do not (Nishimori et al. 2009a, c). This implies that the hepatic toxicity of silica nanoparticles has a specific threshold determined by the grain diameter. In this study, to examine the effects of silica nanoparticles in liver injury, silica nanoparticles of a diameter of 100 nm were used. In addition, we examined the mechanism by which silica nanoparticles caused liver injury, and investigated the synergistic effects on hepatic toxicity of SP100 with pharmaceutical agents.

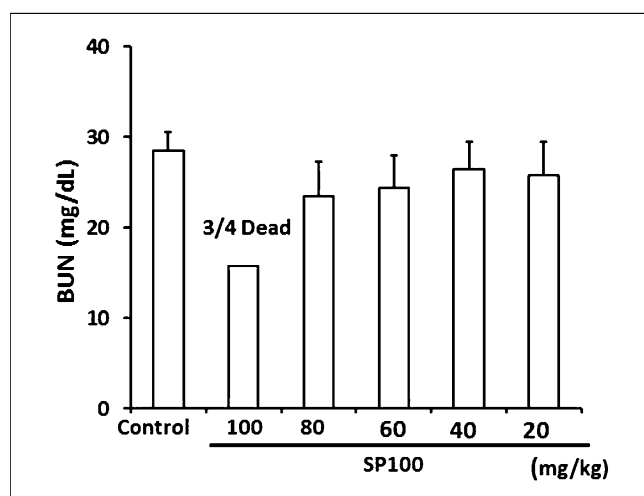
2. Investigations, results and discussion

2.1. *Acute toxicity of 100-nm-diameter silica nanoparticles*

We initially examined liver injury caused by silica nanoparticles with diameters of 100 nm (SP100), and investigated the acute liver toxicity of silica nanoparticles with diameters of 100 nm at a maximal dose of 100 mg/kg (Fig. 1). Acute liver toxicity of SP100 rose dose-dependently (Fig. 1A). Intravenous injection of SP100 at 100 mg/kg was often lethal in mice. There was acute liver toxicity of SP100 at 60 mg/kg. Moreover, elevation of blood urea nitrogen, a biochemical marker of kidney injury, was not observed (Fig. 1B). These data demonstrated that silica nanoparticles caused acute liver toxicity at a diameter of 100 nm. Next, SP100 at doses of 60 mg/kg, SP70 at 40 mg/kg, and SP300 at 100 mg/kg were administered to mice. As shown in Fig. 2A, at 24 h after silica nanoparticle treatment, the levels of serum ALT after SP70 treatment greatly increased. There was no liver injury caused by SP300, though there was liver injury caused by SP100. Figure 2 (B-E) shows hematoxylin-eosin staining of the liver in silica nanoparticle-injected mice. Liver injury was confirmed in SP70 and SP100 treated mice, but not in SP300 treated mice. These results show that silica nanoparticles with a diameter of 100 nm and below cause liver toxicity.



(A)



(B)

Fig. 1: Dose-dependency of SP100 on liver and kidney injury SP100 was intravenously administered at the indicated doses. Serum ALT, AST, (A) and BUN (B) at 24 h were measured using a commercially available kit, as described in the Experimental section. Data are means ± SEM (n = 4). *Significantly different compared with the vehicle-treated group ($p < 0.05$).

Nanosized particles are defined as having a grain diameter of 100 nm or less. Our results showed that there was hepatic toxicity caused by SP100 and SP70, but not by SP300. Vamanu et al. (2008) reported that TiO_2 nanoparticles of a diameter of 100 nm or less are cytotoxic. Additionally, Shavandi et al. (2010) reported that silver nanoparticles of a diameter of 100 nm or less are cytotoxic. These results show that the grain diameter is critical for determining the level of toxicity to the body and to cells, and in particular, they imply that toxicity is induced by particles of 100 nm or less.

The acute liver toxicity of SP100 increased in a dose-dependent manner (Figs. 1, 2). Moreover, we assessed the presence of liver fibrosis in SP100 treatments. SP100 significantly increased the hepatic hydroxyproline content (data not shown). We previously found that SP70 causes acute liver toxicity and hepatic fibrosis (Nishimori et al. 2009a, c). In the present study, we found that SP70 had a larger effect on liver damage compared to SP100 (Fig. 2). These results show that the level of hepatic toxicity changes according to the grain diameter of the silica particles. In future studies, it will be necessary to examine the level of hepatic toxicity caused by a wide variety of grain diameters of silica nanoparticles.

2.2. Mechanism of acute liver toxicity by silica nanoparticles

We investigated the liver toxicity caused by silica nanoparticles when combined with agents that inhibit the activities of liver sinusoidal endothelial cells or liver Kupffer cells. Cyclophosphamide (CPA) is an alkylating agent that induces apoptosis in liver sinusoidal endothelial cells (DeLeve 1996; Malhi et al. 2002). $GdCl_3$ inhibits phagocytosis by Kupffer cells and transiently eliminates them, and $GdCl_3$ has been widely used to investigate the roles of Kupffer cells in the liver (Hardonk et al. 1992; van Til et al. 2005). We thus investigated the effects of CPA and $GdCl_3$ on silica nanoparticle-induced liver injury. As shown in Fig. 3A, pre-injection of CPA did not affect the ALT levels in mice, whereas in silica nanoparticle-injected mice, CPA dramatically decreased ALT levels to near control values. Moreover, as shown in Fig. 3B, pre-injection of $GdCl_3$ prior to injection of silica nanoparticles elevated serum ALT levels 2-fold or more in the silica nanoparticle-injected group. Next, we investigated the cytotoxicity of SP70, SP100, and SP300 in primary cultured hepatocytes isolated from mice. SP70 and SP100 at 100 μg/ml were toxic to primary hepatocytes to almost the same degree, indicating that the differences in liver injury among these nanoparticles were not due to differences in the sensitivity of hepatocytes to the nanoparticles (Fig. 3C). These data indicated that liver sinusoidal endothelial cells are directly involved in silica nanoparticle-induced liver injury, and that phagocytosis of silica nanoparticles by Kupffer cells attenuates liver injury.

Liver sinusoidal endothelial cells form the basic tubular vessels for transvascular exchange between the blood and the surrounding tissue (McCuskey 2008). Kupffer cells are a component of the sinusoidal wall and play a significant role in the removal of particles and cells as well as toxic substances (Wisse et al. 1996). We observed that the ALT values decreased to near control levels by the administration of silica nanoparticles and CPA (Fig. 3A). This indicates that liver sinusoidal endothelial cells greatly influence the hepatic toxicity by silica nanoparticles. There are several receptors on the cell surface of liver sinusoidal endothelial cells, and it is known that they endocytose proteins and large particles (Smedsrod et al. 1997; Steffan et al. 1986). It is likely that silica nanoparticles are englobed by liver sinusoidal endothelial cells, after which they accumulate and are then discharged into hepatocytes. A detailed analysis of the relationship between liver sinusoidal endothelial cells and silica nanoparticles is necessary for future studies.

2.3. Influence of 100-nm-diameter silica nanoparticles with cisplatin or paraquat-induced toxicity

Previously, we reported synergistic toxicity of SP70 with CDDP and PQ (Nishimori et al. 2009b). CDDP is widely used as an anti-tumor agent, and PQ is one of the most widely used and highly toxic herbicides (Ozols and Young 1991; Vandenbogaerde et al. 1984; Witjes 1997). In the present study, we thus investigated the synergistic effects of hepatic toxicity of SP100 with CDDP or PQ. To avoid direct interactions between the chemicals and SP100 before administration and absorption, we injected the chemicals and SP100 intraperitoneally and intravenously, respectively. We observed that the serum levels of ALT were elevated by CDDP (Fig. 4A). We next investigated the interaction between PQ and SP100. Co-administration of PQ and SP100 elevated levels of serum ALT, and SP100 showed synergistic elevation of serum ALT levels from 138.2 to 492.0 KU (Fig. 4B).

We investigated the combined effects of the chemicals on nanoparticle-induced toxicity, and found that CDDP and PQ had synergistic toxic effects with SP100. Verma et al. reported

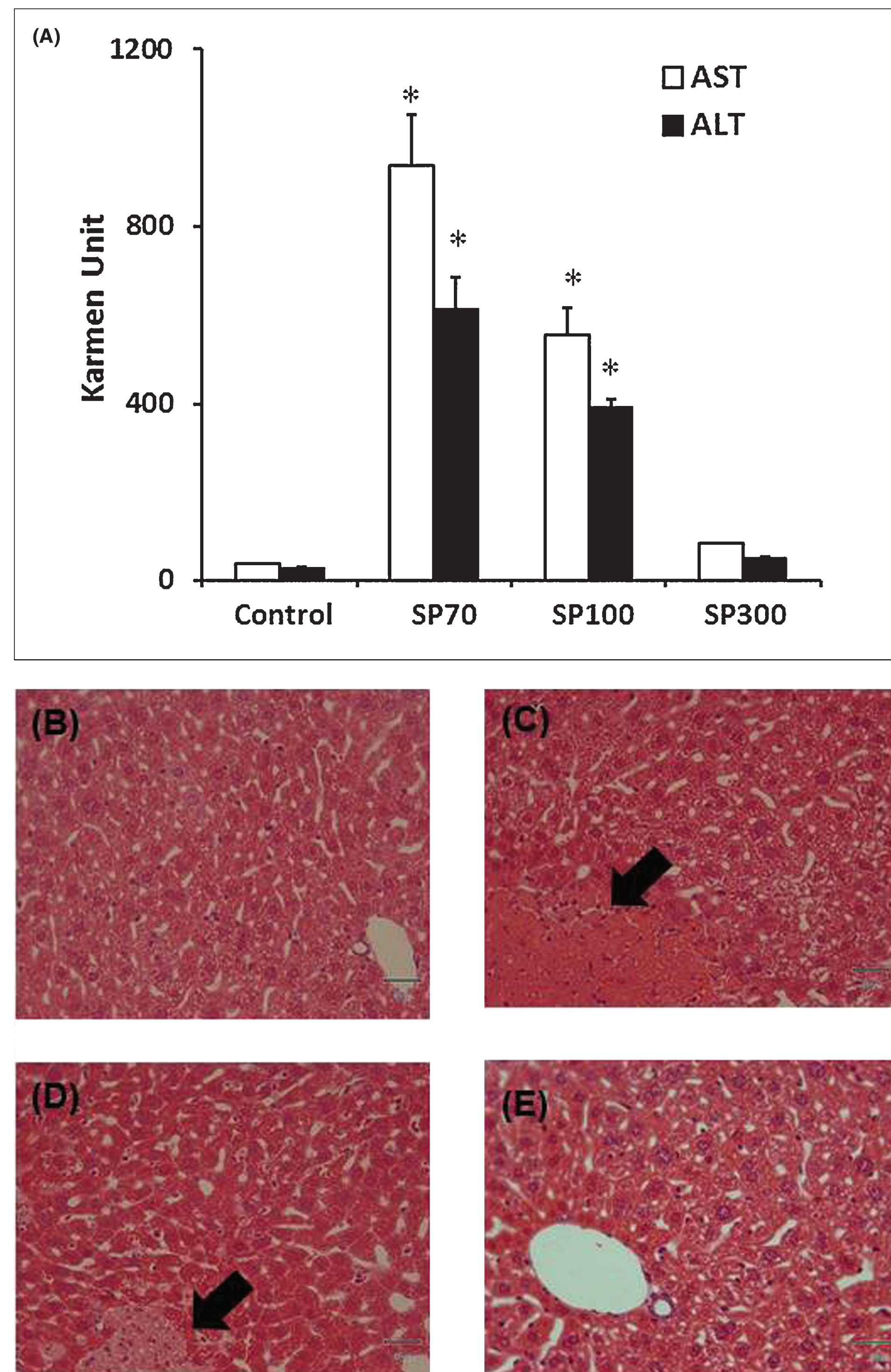


Fig. 2: Comparison of acute liver toxicity of silica nanoparticles Serum ALT and AST (A) at 24 h were measured using a commercially available kit as described in the Experimental section. Histological analysis of silica nanoparticle-treated mice were conduced on tissues fixed with 4% paraformaldehyde 24 h after administration of vehicle (B), SP70 (C), SP100 (D), and SP300 (E). 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 h after injection. The arrow shows hepatic injury. Data are means ± SEM (n = 4). *Significantly difference compared with the vehicle-treated group ($p < 0.05$).

that the blood circulation levels of CDDP are made to rise by nanomaterial conjugates of CDDP (Verma and Saching 2008). In addition, Moreno et al. (2009) reported that PLGA nanoparticles improve the effects of CDDP. Therefore, nanoparticles could possibly increase both the beneficial effects and toxicity of chemicals and drugs. Further evaluation of such interactions between nanomaterials and pharmaceutical agents for future pharmaceutical applications are necessary.

This report is the first to show that silica nanoparticles with a diameter of 100 nm or less have hepatic toxicity, and that liver injury is mediated by liver sinusoidal endothelial cells. Further studies based on these data should provide useful information regarding the safety of nanomaterials.

3. Experimental

3.1. Materials

Silica particles with a diameter of 70, 100, or 300 nm (SP70, SP100, SP300) were obtained from Micromod Partikeltechnologie GmbH (Rostock,

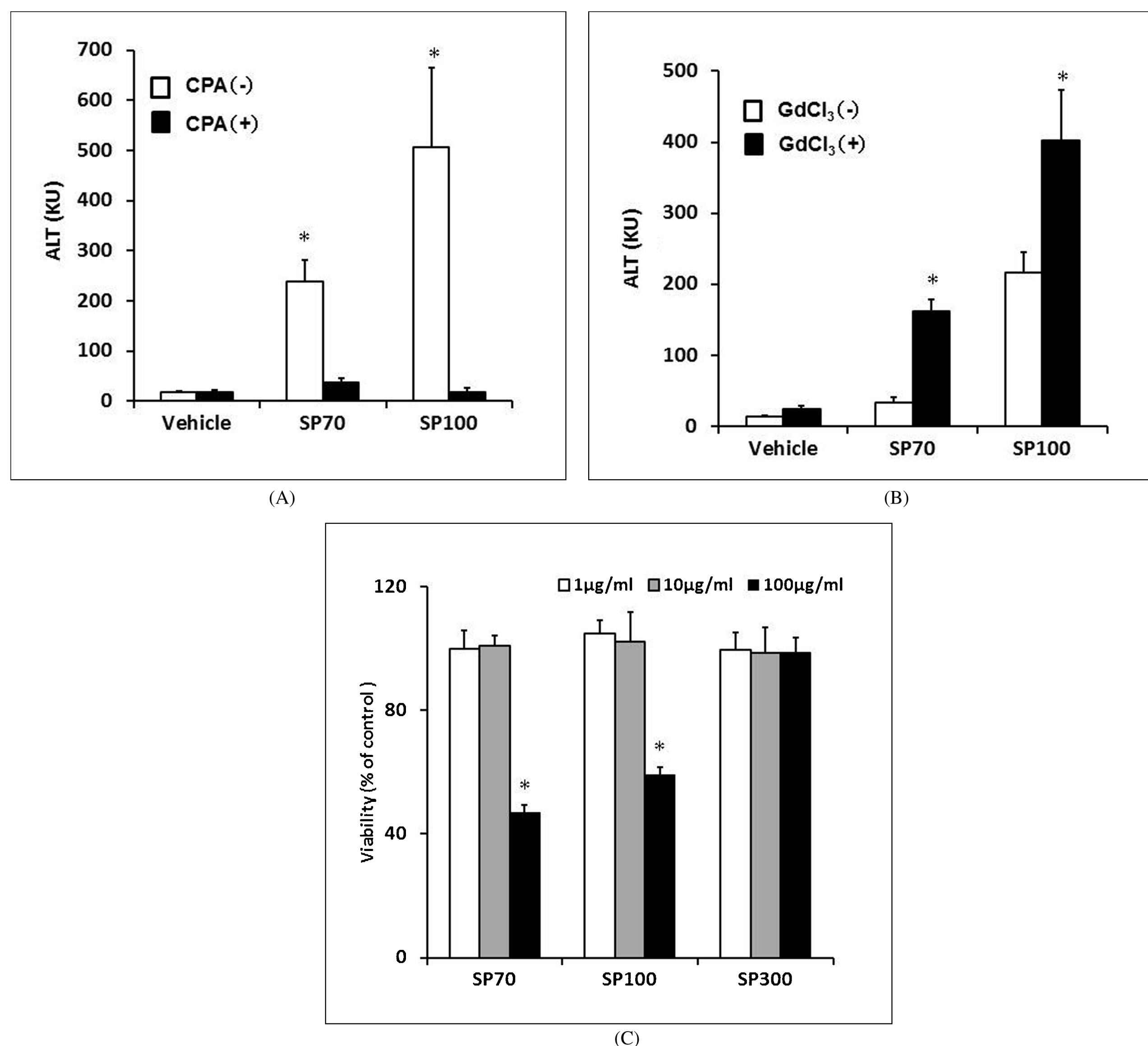


Fig. 3: Analysis of silica nanoparticle-induced liver injury Effect of CPA (A). Vehicle or CPA (300 mg/kg) was intraperitoneally injected into mice 24 h prior to treatment with silica particles (SP70, 40 mg/kg; SP100, 60 mg/kg). At 24 h after administration of particles, blood was recovered, and the resultant serum was used for ALT assay. Data are means ± SEM (n = 4). *Significant difference between vehicle and silica particle-treated groups ($p < 0.05$). Effect of $GdCl_3$ (B). Vehicle or $GdCl_3$ (10 mg/kg) was intravenously injected into mice at 30 h or 6 h prior to treatment with silica particles. 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 $GdCl_3$ + and $GdCl_3$- groups ($p < 0.05$). (C) Comparison of cytotoxicity of silica nanoparticles in primary hepatocytes. Hepatocytes were prepared from mouse livers by the collagenase perfusion method, as described in the Experimental section. Cells were seeded onto 96-well plates at 5×10^3 cells/well, and were treated with silica particles at the indicated concentrations. After 48 h of treatment, viability was measured by WST-8 assay as described in the Materials and methods. Data were normalized against vehicle-treated cells (100% control). Data are means ± SD (n = 4).

Germany). The size distribution of the particles was analyzed using a Zetasizer (Sysmex Co., Kobe, Japan), and the mean diameters were 57.5 ± 20.3, 137 ± 32.1, and 296 ± 36.3 nm, respectively. The particles were spherical and nonporous and were stored at 25 or 50 mg/mL in an aqueous suspension. The suspensions were thoroughly dispersed with sonication before use and then diluted in ultrapure water. Paraquat (PQ) and cisplatin (CDDP) were dissolved in saline and stored at −20 °C before use. All reagents used were of research grade.

3.2. Animals

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were maintained in a controlled environment (23 ± 1.5 °C; 12-h light/dark cycle) with access to standard rodent chow and water *ad libitum*. The mice were left to adapt to the new environment for 1 week before commencing with the experiment. Mice that received a single treatment of silica nanoparticles were anesthetized and sacrificed 24 h after intravenous injection. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

3.3. Biochemical analysis

Serum alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured with commercially available kits according to the manufacturer's protocols (Wako Pure Chemical Industries, Osaka, Japan).

3.4. Histological analysis

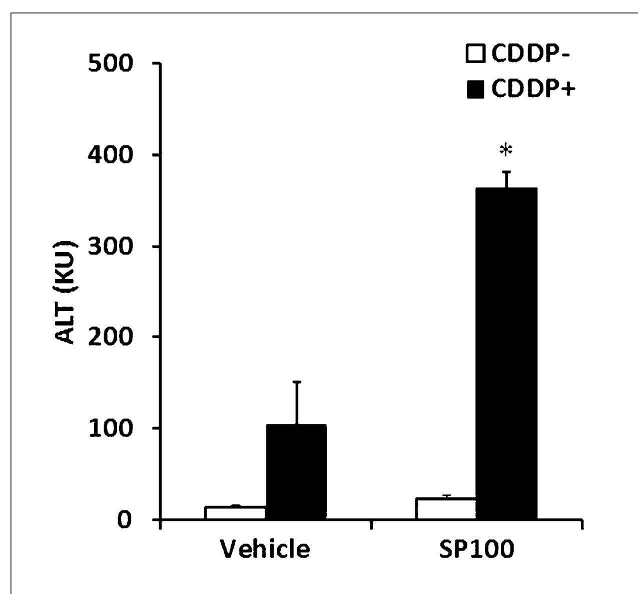
The liver was removed and fixed with 4% paraformaldehyde. After sectioning, thin tissue sections were stained with hematoxylin and eosin for histological observation.

3.5. Cyclophosphamide assay

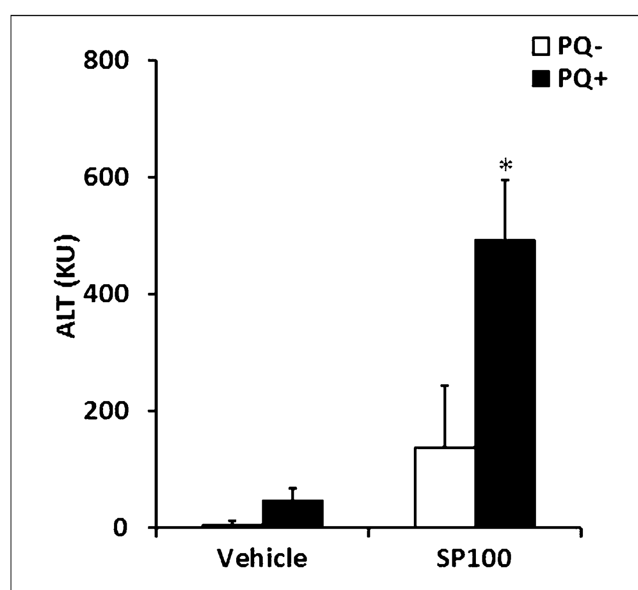
Disruption of liver sinusoidal endothelial cells was carried out by intraperitoneal injection of 300 mg/kg body weight cyclophosphamide (CPA) at 24 h prior to administration of nanoparticles. Blood was recovered at 24 h after injection of nanoparticles for the ALT assay.

3.6. 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



(A)



(B)

Fig. 4: Effect of SP100 on cisplatin- and paraquat-induced toxicity (A) Mice were injected with cisplatin (CDDP) at 0 or 50 μmol/kg and SP100 at 10 mg/kg, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT levels were assayed as described in the Experimental section. Data are means ± SEM (n = 4). *Significant difference between vehicle and CDDP-treated group ($p < 0.05$). (B) Mice were injected with paraquat (PQ) at 0 or 50 mg/kg and SP100 at 60 mg/kg, intraperitoneally and intravenously, respectively. At 24 h post-injection, the serum was recovered. ALT levels were assayed as described in the experimental section. Data are means ± SEM (n = 4). *Significant difference between vehicle and PQ-treated group ($p < 0.05$).

10 mg/kg body weight at 30 h and 6 h prior to intravenous administration of nanoparticles. Blood was then recovered 24 h after injection of nanoparticles for the ALT assay.

3.7. Cytotoxicity in primary hepatocytes

Hepatocytes were isolated from BALB/c mice by Seglen's method using perfusion of collagenase (Seglen 1976). Viability of the isolated hepatocytes was assayed by trypan blue staining, and cells (over 90% viability) were seeded onto 96-well plates at 5×10^3 cells/well, and the cells were treated with silica particles for 48 h. Cell viability was then assayed with the Cell Counting Reagent SF, according to the manufacturer's protocol (Nacalai Tesque, Kyoto Japan).

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