

## Liver Injury Induced by Thirty- and Fifty-Nanometer-Diameter Silica Nanoparticles

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**Nano-size silica material is a promising reagent for disease diagnosis, cosmetics, and the food industry. For the successful application of nanoparticle materials in bioscience, evaluation of nano-size material toxicity is important. We previously found that nano-size silica particles caused acute liver failure in mice. However, the hepatotoxicity of nanosilica particles with the diameter of 70 nm or less is unknown. Here, we investigated the relationship between particle size and toxicity using nanosilica particles with diameters of 30, 50, and 70 nm (SP30, SP50, and SP70, respectively). We observed dose-dependent increases in hepatic injury following administration of SP50 and SP30, with SP30 causing greater acute liver injury than that seen with SP50. Smaller silica nanoparticles induced liver injury even at proportionally lower dose levels. Furthermore, we investigated the combinatorial toxicity of SP30 in the presence of chemically induced liver injury (including that caused by carbon tetrachloride, paraquat, cisplatin, and acetaminophen). We observed that particles of the smallest size tested (SP30) synergized with chemical substances in causing liver injury. These data suggest that the size (diameter) of the silica nanoparticles affects the severity of nanoparticle-induced liver injury, a finding that will be useful for future investigations in nanotechnology and nanotoxicology.**

**Key words** silica nanoparticle; liver injury; paraquat; cisplatin; acetaminophen

Recently, the proposed scientific, medical, and technical applications of nanomaterials have greatly increased. Nanomaterials are frequently used in microelectronics, cosmetics, and sunscreen, and their potential use in drug-delivery systems is being investigated.<sup>1–3)</sup> Nanomaterials have unique physicochemical qualities compared to micromaterials with regard to size, surface structure, solubility, and aggregation. Thus, the reduction in particle size from the micro- to nanoscale may be beneficial for many industrial and scientific applications. However, nanomaterials have potential toxicities not found in micromaterials, making it essential to understand the biological activity and potential toxicity of nanomaterials.<sup>4,5)</sup>

Previously, we reported that silica nanoparticles with diameters of 70-nm (SP70) or 100-nm (SP100) were associated with liver injury; this hepatotoxicity was not seen with 300-nm-diameter silica nanoparticles (SP300).<sup>6,7)</sup> Silica nanoparticles are being increasingly used in cosmetics and for the systemic and local delivery of drugs, and the production of silica nanoparticles currently exceeds 1350000 tons per year worldwide.<sup>8,9)</sup> Given that many products use silica nanoparticles with diameters of 10 nm or less, it is critical to investigate the correlation between nano-size and the hepatic toxicity of silica nanoparticles.

Nano-materials also are used in food and medical supplies,<sup>1,10)</sup> items that are intended for *in vivo* use. Indeed, nanomaterials have been proposed as components of tumor-targeting therapies and biomedical imaging reagents.<sup>11,12)</sup> In previous work, we reported that the SP70 and SP100 silica nanoparticles induced liver injury by drug interaction.<sup>13,14)</sup> However, to our knowledge, there are no reports of drug interactions by silica nanoparticles with diameters of less than 70 nm. It thought that the hepatic toxicity of silica nanoparticles reflects a threshold of sensitivity based on grain diameter. Therefore,

to examine the role of particle diameter on liver injury, we examined the effects of silica nanoparticles at diameters of 30 nm (SP30) and 50 nm (SP50). We additionally tested potential synergistic effects on liver toxicity of SP50 and SP30 in combination with known hepatotoxins.

### MATERIALS AND METHODS

**Materials** Silica particles with diameters of 30, 50, 70, 300, or 1000 nm were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The size distribution of the particles was analyzed by Zetasizer (Sysmex Co., Kobe, Japan), and mean diameters were 27.4, 46.3, 55.7, 296, and 989 nm, respectively. The particles, which were spherical and nonporous, were formulated and stored at 25 mg/mL (30, 50, 70 nm) and 50 mg/mL (300, 1000 nm) in aqueous suspension. Prior to each administration, the suspensions were thoroughly dispersed by sonication and diluted with water. Identical volumes of suspension were injected in each experiment. Paraquat, cisplatin and acetaminophen were dissolved in saline and stored at –20°C until use. All reagents used were research grade.

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

**Biochemical Analysis** Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and blood

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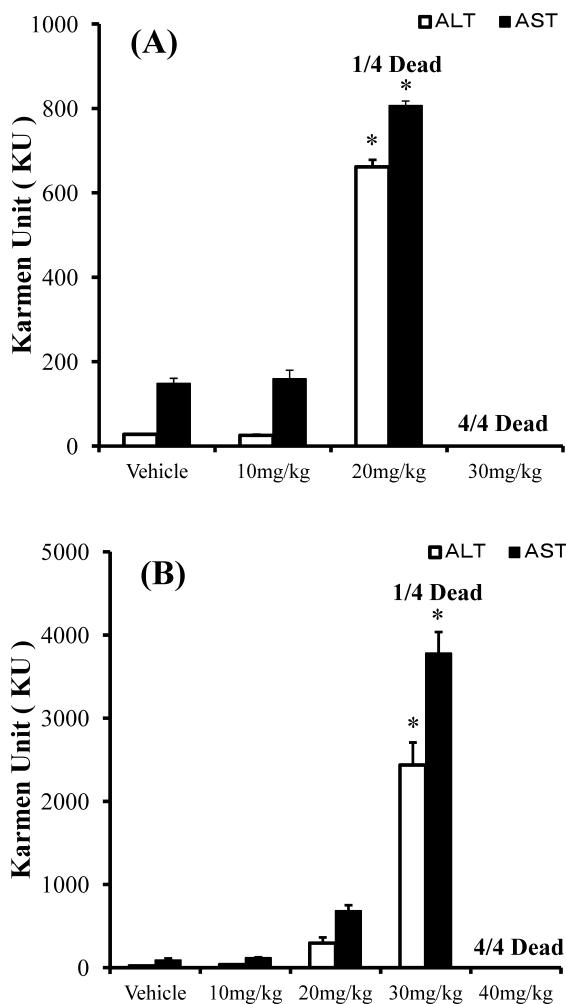


Fig. 1. Dose Dependency of SP30- and SP50-Induced Liver Injury

Serum levels of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a commercially available kit (see Materials and Methods) at 24 h after intravenous (i.v.) administration of SP30 (A) and SP50 (B) at the indicated doses. Data are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ). \*Significant difference ( $p < 0.05$ ) compared to the vehicle-treated group.

urea nitrogen (BUN) were measured using commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocols.

**Histological Analysis** Histological analysis of liver from silica nanoparticle-treated mice. At 24 h after IV administration with vehicle or with 30 mg/kg SP30, SP50, or SP70, fixed with 4% paraformaldehyde. Following processing and sectioning, thin tissue sections were stained with hematoxylin and eosin for histological observation.

**Statistical Analysis** Statistical analyses were performed using two-way ANOVA, followed by Student's *t*-test.  $p < 0.05$  was considered statistically significant.

## RESULTS

**Acute Toxicity of Diameter 30- and 50-nm Silica Nanoparticles** We initially examined the liver injury following the intravenous (i.v.) administration of SP30 and SP50 silica nanoparticles at concentrations of up to 40 mg/kg (Figs. 1A,B). Notably, SP30 administered at 20 or 30 mg/kg resulted in (respectively) 25% (1 of 4 mice) or 100% (4 of 4) mortality

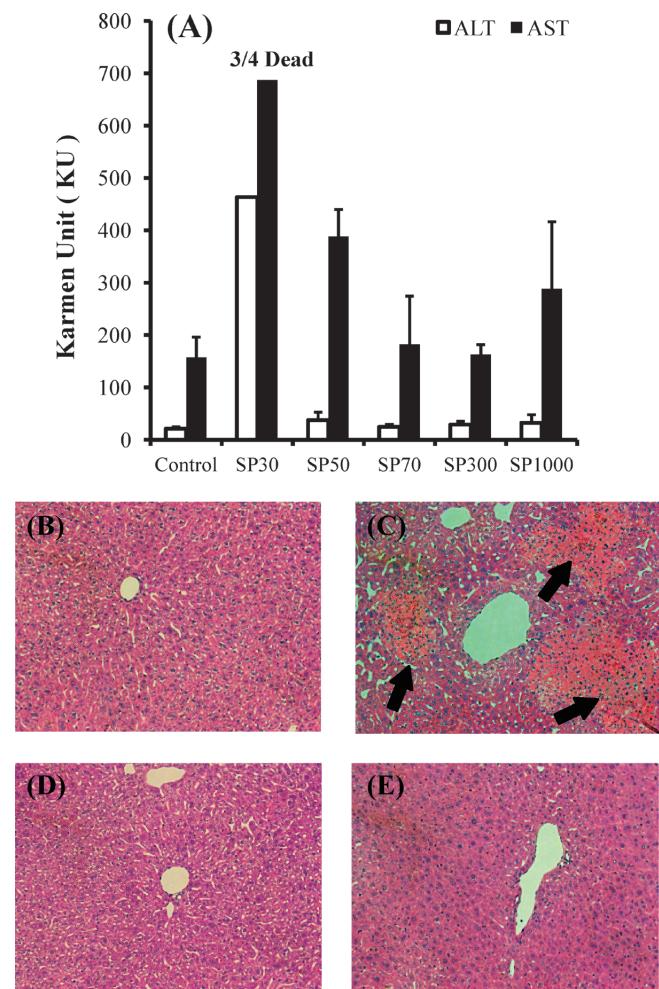


Fig. 2. Comparison of Acute Liver Toxicity of Silica Nanoparticles

(A) Serum levels of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a commercially available kit (see Materials and Methods) at 24 h after intravenous (i.v.) administration of nanoparticles of the indicated size at 30 mg/kg (SP30, SP50, and SP70) or at 100 mg/kg (SP300 and SP1000). Data are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ), except for SP30-dosed animals (1 survivor). (B to E) Histological analysis of liver from silica nanoparticle-treated mice. At 24 h after i.v. administration with vehicle (B) or with 30 mg/kg SP30 (C), SP50 (D), or SP70 (E), tissues were collected, fixed with 4% paraformaldehyde, sectioned, and stained with hematoxylin and eosin. Arrows designate sites of hepatic injury.

within 24 h. SP50 administered at 30 or 40 mg/kg resulted in (respectively) 25% (1 of 4 mice) or 100% (4 of 4) mortality within 24 h. The surviving animals exhibited acute liver toxicity (as indicated by elevated ALT and/or AST levels), with an apparent dose-dependent effect (Figs. 1A,B). These animals did not exhibit elevations of blood urea nitrogen, a biochemical marker of kidney injury (data not shown). These data demonstrated that the hepatic toxicity of silica nanoparticles increased with decreasing particle diameter.

To further assess the comparative toxicity of nanoparticles of different sizes, we administered SP30, SP50, and SP70 (at 30 mg/kg), and SP300 and SP1000 (at 100 mg/kg) i.v. in mice. As shown in Fig. 2A, 24 h after silica nanoparticle treatment, SP30 at 30 mg/kg was lethal in three of four mice; further, the activity of serum ALT was greatly increased in the surviving mouse. Intermediate increases in ALT also were seen in mice that received SP50. On histologic evaluation, the surviving SP30 mouse exhibited microscopic signs of liver injury; such signs were not seen in the animals that received SP50, SP70

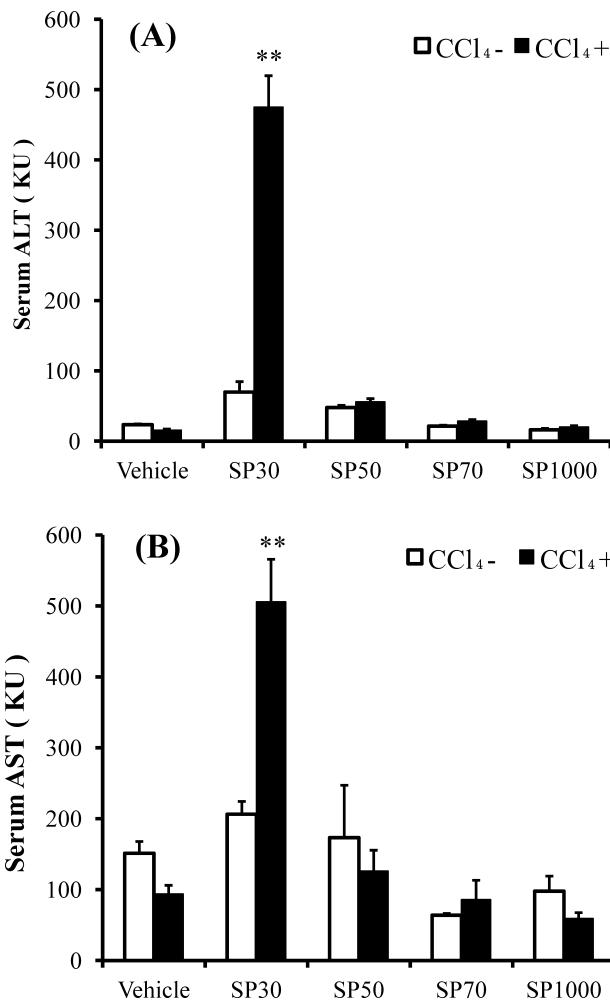


Fig. 3. Effect of SP30 on Carbon Tetrachloride-Induced Toxicity

Mice were injected intraperitoneally with carbon tetrachloride ( $\text{CCl}_4$ ) at 0 (open column) or 0.01 mL/kg (solid column) together with intravenous injection of vehicle or silica nanoparticles (15 mg/kg) of the indicated size. At 24 h post-injection, serum levels of liver enzymes alanine aminotransferase (ALT; Panel A) and aspartate aminotransferase (AST; Panel B) were determined using a commercially available kit (see Materials and Methods). Data are representative of three independent experiments, and are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ). \*\*Significant difference ( $p<0.01$ ) between vehicle- and  $\text{CCl}_4$ -treated group.

(Figs. 2B–E). These data demonstrated that i.v. injection of SP30 causes greater acute liver injury than does SP50.

**Influence of Diameter 30-nm Silica Nanoparticles on Chemically Induced Hepatotoxicity** Next, we investigated whether there are combinatorial effects of hepatic injury caused by chemicals and the nanoparticles. To avoid interactions between the chemicals and silica nanoparticles (e.g., absorption) prior to administration, the reagents were administered separately, with the chemicals injected intraperitoneally (i.p.) and the silica nanoparticles injected i.v. For this experiment, the nanoparticles were administered at dose levels (15 mg/kg for SP30, SP50, and SP70; 100 mg/kg for SP1000) below those known to cause acute lethality.

While carbon tetrachloride ( $\text{CCl}_4$ ) is known to induce hepatic injury following i.p. administration,<sup>15</sup> we administered  $\text{CCl}_4$  (0.01 mL/kg) at a dose that does not induce hepatic injury when administered alone (Figs. 3A,B). Co-administration of  $\text{CCl}_4$  and SP30 resulted in increased ALT and AST levels with other sizes of nanoparticles, changes in enzyme levels were not significant.

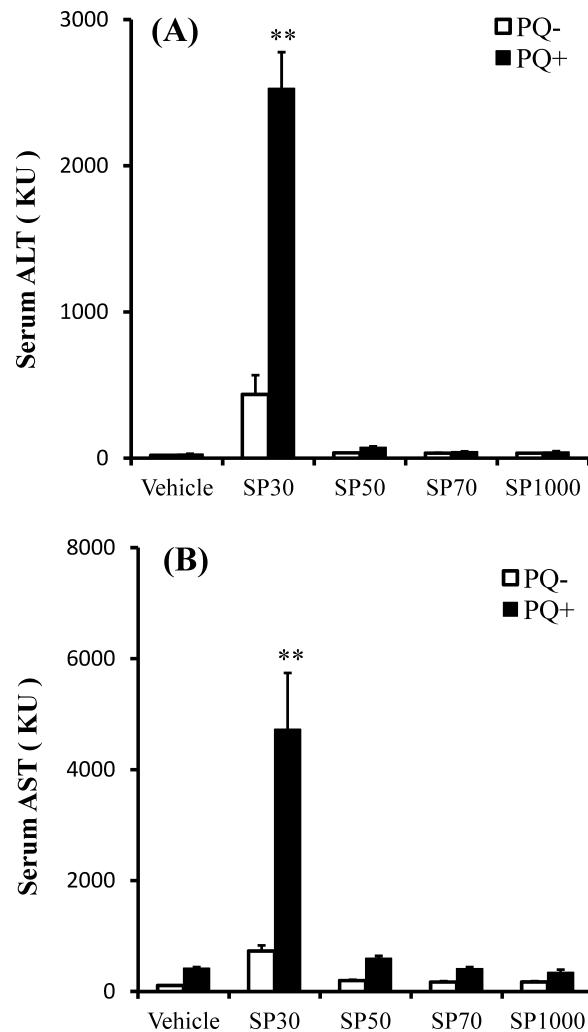


Fig. 4. Effect of SP30 on Paraquat-Induced Toxicity

Mice were injected intraperitoneally with paraquat (PQ) at 0 (open column) or 50 mg/kg (solid column) together with intravenous injection of vehicle or silica nanoparticles (15 mg/kg) of the indicated size. At 24 h post-injection, serum levels of liver enzymes alanine aminotransferase (ALT; Panel A) and aspartate aminotransferase (AST; Panel B) were determined using a commercially available kit (see Materials and Methods). Data are representative of three independent experiments, and are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ). \*\*Significant difference ( $p<0.01$ ) between vehicle- and PQ-treated group.

Previously, we reported the synergistic toxicity of SP70 with cisplatin (CDDP) or paraquat (PQ).<sup>16</sup> PQ is a widely used and highly toxic herbicide.<sup>17,18</sup> We therefore investigated the synergistic effects on hepatic toxicity of SP30 with paraquat (50 mg/kg). As seen above for carbon tetrachloride, co-administration of PQ and SP30 resulted in increased serum levels of ALT and AST (Figs. 4A,B). Similar synergy was not seen with larger-diameter nanoparticles.

Next, we tested the potential interaction between CDDP (a widely used anti-tumor agent)<sup>18</sup> and silica nanoparticles (Figs. 5A–C). Notably, all (4 of 4) of the mice that received both CDDP and SP30 died within 24 h of dosing, although the mice administered either of these reagents alone did not exhibit such mortality. Co-administration of CDDP and SP50 resulted in elevations in markers of both liver (ALT, AST) and kidney (BUN) damage (Figs. 5A–C). Changes for these markers were not significant for combinations of CDDP with larger-diameter nanoparticles.

Finally, we investigated the interaction between acetamino-

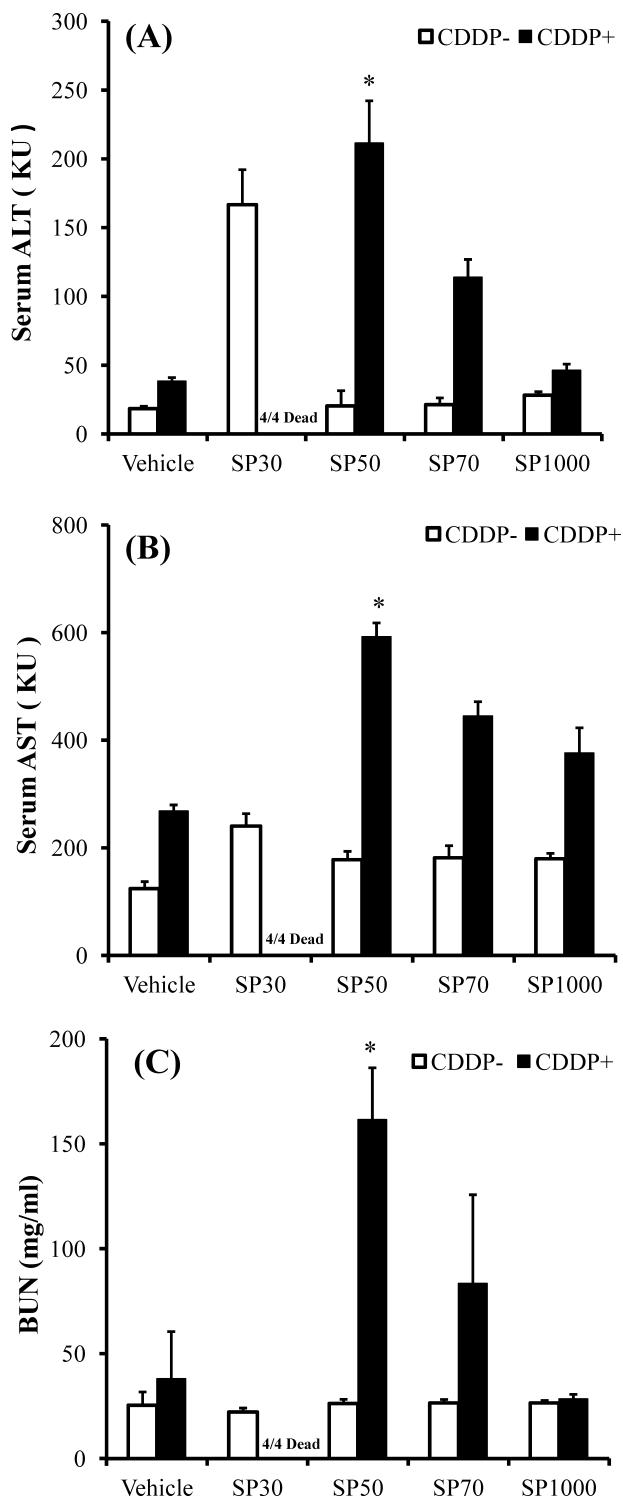


Fig. 5. Effect of SP30 on Cisplatin-Induced Toxicity

Mice were injected intraperitoneally with cisplatin (CDDP) at 0 (open column) or 100  $\mu\text{mol}/\text{kg}$  (solid column) together with intravenous injection of vehicle or silica nanoparticles (15 mg/kg) of the indicated size. At 24 h post-injection, serum levels of liver enzymes alanine aminotransferase (ALT; Panel A) and aspartate aminotransferase (AST; Panel B), and plasma levels of blood urea nitrogen (BUN; Panel C) were determined using a commercially available kit (see Materials and Methods). Data are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ). \*Significant difference ( $p < 0.05$ ) between vehicle- and CDDP-treated group.

phen (AA, a non-steroidal anti-inflammatory drug) and silica nanoparticles (Figs. 6A, B). Co-administration of AA with SP30 caused significant elevations in serum liver enzymes.

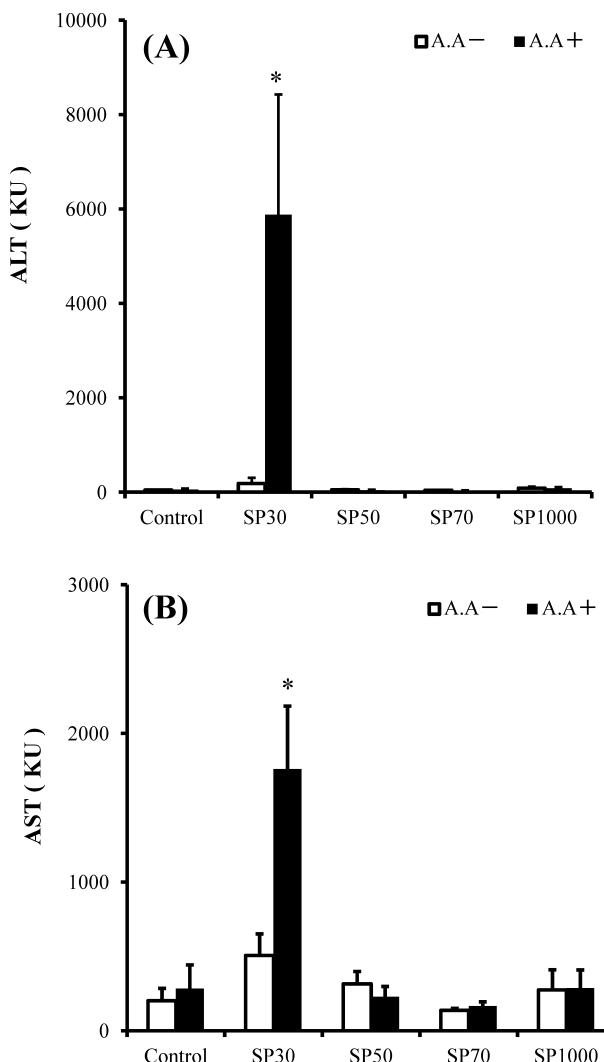


Fig. 6. Effect of SP30 on Acetaminophen-Induced Toxicity

Mice were injected intraperitoneally with acetaminophen (AA) at 0 (open column) or 400 mg/kg (solid column) together with intravenous injection of vehicle or silica nanoparticles (15 mg/kg) of the indicated size. At 24 h post-injection, serum levels of liver enzymes alanine aminotransferase (ALT; Panel A) and aspartate aminotransferase (AST; Panel B), and plasma levels of blood urea nitrogen (BUN; Panel C) were determined using a commercially available kit (see Materials and Methods). Data are presented as mean  $\pm$  standard error of the mean (S.E.M.;  $n=4$ ). \*Significant difference ( $p < 0.05$ ) between vehicle- and AA-treated group.

Changes for ALT and AST were not significant for combinations of AA with larger-diameter nanoparticles.

## DISCUSSION

In the present study, we evaluated the toxicity of silica nanoparticles with diameters of 30 (SP30) and 50 nm (SP50). We found that the smaller-diameter nanoparticles induced lethality and/or liver injury when administered alone (at doses of 15 mg/kg or higher), and displayed synergistic effects on hepatic toxicity when administered at a sub-lethal dose in combination with a sub-acute dose of known hepatotoxin.

In comparing the effects of nanoparticles at a range of sizes, we observed an inverse correlation between hepatic toxicity and particle diameter. Specifically, we observed that SP30 and SP50 induced murine liver injury at minimum doses of (respectively) 20 and 30 mg/kg. In previous work, we reported

that SP70 induced murine liver injury at a dose of 40 mg/kg.<sup>6</sup> Separately, Hirai *et al.* reported that the silica nanoparticles with diameter of 100 nm or less had immune-modulating effects, and that these effects were size-dependent.<sup>19</sup> Moreover, Inoue *et al.* reported that carbon black nanoparticles 14 nm in diameter caused greater acute lung injury than that seen with 56-nm-diameter carbon black nanoparticles.<sup>20</sup> Thus, our results are consistent with previous work, and suggest that multiple toxicities of nanoparticles exhibit an inverse correlation with particle size.

We also investigated the potential synergies of chemical- and nanoparticle-induced hepatotoxicities. As judged by serum levels of liver enzymes ALT and AST, three of the 4 tested hepatotoxins (CCl<sub>4</sub>, PQ, and AA (see below)) exhibited synergistic effects when co-administered with SP30 silica particles. Co-administration of SP30 with CDDP resulted in complete mortality (4 of 4 mice), suggesting a more severe synergy. Previously, we reported that administration of SP70 at 30 mg/kg increased the liver injury associated with administration of PQ or CDDP.<sup>16</sup> In the present study, SP30 demonstrated a similar synergy at a lower dose than that required with SP70. For CCl<sub>4</sub>, PQ, and CDDP, liver injury is associated with the generation of reactive oxygen species (ROS).<sup>21–23</sup> Nabeshi *et al.* previously reported that silica nanoparticles induce ROS production by HaCata cells growing *in vitro*.<sup>24</sup> In the context of the current work, we presume that SP30 produces ROS in liver, providing a synergistic interaction with the liver injury caused by CCl<sub>4</sub>, PQ, and CDDP. In the future, we propose to measure the concentration of ROS in the liver following administration of nanosilica particles.

In this study, co-administration of AA and SP30 resulted in hepatic damage, as indicated by increased serum ALT and AST levels. It has been reported that AA can induce severe hepatic damage,<sup>25,26</sup> a side effect that reflects exhaustion of glutathione in the liver.<sup>27,28</sup> Ahmad *et al.* reported that the silica nanoparticles induced depletion of glutathione in human liver cell line HepG2.<sup>29</sup> We propose that the synergistic liver injury seen for co-administration of AA and SP30 reflects the shared depletion by these reagents of the glutathione pool in hepatocytes.

In summary, we have demonstrated that 30-nm-diameter silica nanoparticles can cause liver damage, an effect that synergizes with known chemical inducers of hepatotoxicity. In general, we observed increasing toxicity of nanoparticles at smaller particle size. Further studies based on these data will be required to assess the toxicological profile of nanoparticles proposed for use in humans.

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