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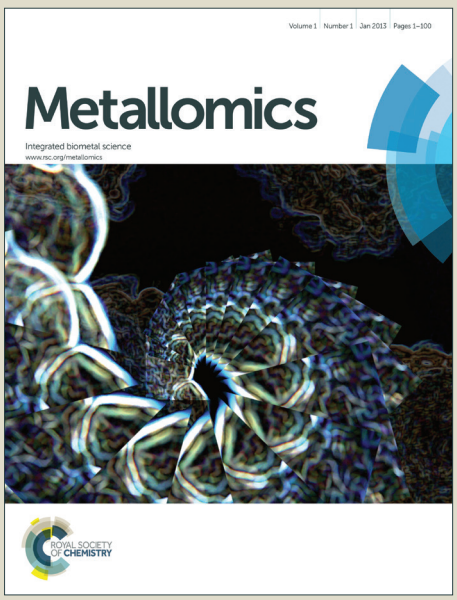

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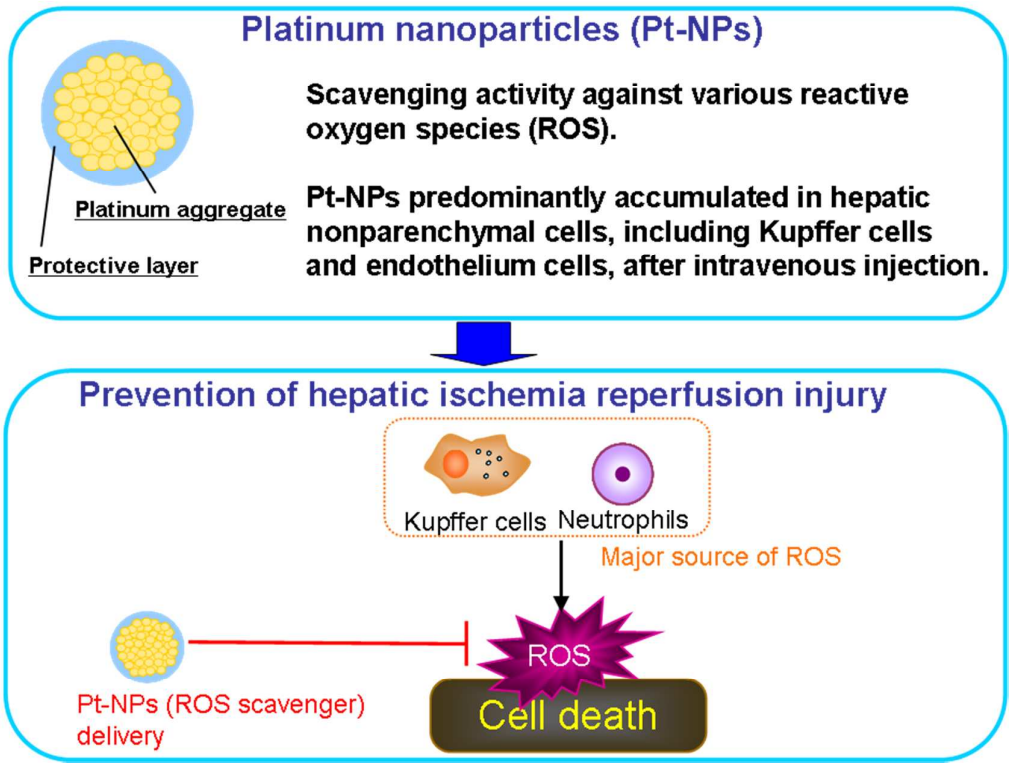
Graphical abstract

We successfully developed platinum nanoparticles (Pt-NPs), which exhibited reactive oxygen species scavenging activity and selective delivery to the liver. Pt-NPs effectively suppressed the hepatic injury in mice following hepatic ischemia/reperfusion.



Pharmacokinetics and preventive effects of platinum nanoparticles as reactive oxygen species scavengers on hepatic ischemia/reperfusion injury in mice

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Abstract

Reactive oxygen species (ROS) are involved in the pathophysiology of ischemia/reperfusion injury. To protect mouse hepatocytes from ischemia/reperfusion injury, we prepared two different sizes of citric acid-protected platinum nanoparticles (Pt-NPs), which exhibited ROS-scavenging activities and selective delivery to a specific type of liver cell. Small Pt-NPs (30 nm) reduced the superoxide anion, hydrogen peroxide, and hydroxyl radical levels in solution to a greater extent than did large Pt-NPs (106 nm). Large and small Pt-NPs predominantly accumulated in hepatic nonparenchymal cells after intravenous injections in mice. In a mouse model of ischemia/reperfusion injury, in which hepatic injury was induced by occluding the portal vein for 15 min followed by 6-h reperfusion, the increase in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities was inhibited by a bolus intravenous injection of either large or small Pt-NPs. However, small Pt-NPs inhibited the increase in these markers of hepatic injury to a greater extent than did large Pt-NPs. These results indicate that Pt-NPs can be used to prevent hepatic ischemia/reperfusion injury. To our knowledge, this is the first report demonstrating the pharmacokinetics and efficacy of Pt-NPs to prevent hepatic ischemia/reperfusion injury.

Introduction

Hepatic ischemia/reperfusion occurs in a variety of clinical settings, such as in shock, transplantation, and liver surgery, and results in severe injuries that substantially contribute to the morbidity and mortality in such cases [1,2]. Oxidative stress is widely recognized as being involved in the pathogenesis of hepatic ischemia/reperfusion injury. Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in Kupffer cells leads to the generation and accumulation of reactive oxygen species (ROS), which activate the transcription of various genes through the nuclear factor-κB (NF-κB)-mediated pathway [3–6]. Increased expression of interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, IL-8, granulocyte colony-stimulating factor (G-CSF), and inducible nitric oxide synthase (iNOS) may cause severe damage to hepatocytes. Taken together, the evidence indicates that eliminating ROS may be a rational approach for inhibiting hepatic ischemia/reperfusion injury. Therefore, ROS scavengers have been considered as therapeutic agents for hepatic ischemia/reperfusion[7].

To date, the ROS scavengers *N*-acetylcysteine, tocopherol, superoxide dismutase (SOD), and catalase have been examined for prevention of hepatic ischemia/reperfusion injury [8–12]. Recently, platinum nanoparticles (Pt-NPs) have also been studied for their ROS-scavenging properties. It was reported that polyacrylic acid-protected Pt-NPs with

high ratio of electrons catalyze and quench superoxide anions and hydrogen peroxide *in vitro*, thereby mimicking superoxide dismutase and catalase, and that these multi-ROS scavenging activities could have some advantages as far as the treatment of ROS-mediated diseases and injury is concerned[13–17]. Despite some reports on the beneficial effects of Pt-NPs, few studies have examined their pharmacokinetic properties or preventive effects on hepatic ischemia/reperfusion injury. Because it is well known that various kinds of microparticles or nanoparticles are rapidly cleared by scavenging systems in the liver and spleen[18], the tissue distribution of Pt-NPs should be determined and their physicochemical properties and pharmacokinetics optimized to obtain maximum therapeutic benefit.

Therefore, the aim of this study was to examine the tissue distribution and therapeutic potential of Pt-NPs in hepatic ischemia/reperfusion injury. To this end, we prepared citric acid-protected Pt-NPs of two different sizes by reacting platinum hydrochloride ions with ascorbic acid and citric acid. The ROS-scavenging activities and the physicochemical characteristics of the resulting Pt-NPs that determine tissue distribution, such as particle size and electrical charge, were analyzed. We also examined the tissue distribution of Pt-NPs after intravenous injections in mice. Moreover, the therapeutic potential of Pt-NPs was investigated in a mouse model of

hepatic ischemia/reperfusion injury.

Experimental

Animals

Male ddY mice (weighing 25–27 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Experimentation Committee of the Kyoto Pharmaceutical University approved all protocols for animal experiments.

Chemicals

Potassium tetrachloroplatinate (K_2PtCl_4) and L-ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trisodium citrate anhydrous, BES-H_2O_2, trypsin inhibitor obtained from soybean, collagenase type II, nitric acid (60%), hydrogen peroxide (30%), and perchloric acid (60%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydroxyphenyl fluorescein (HPF) was purchased from Sekisui Medical Co., Ltd. (Tokyo, Japan).

2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (CLA) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

Preparation of platinum nanoparticles

Variable particle sizes of Pt-NPs were prepared by reacting different amounts of ascorbic acid and citric acid with K_2PtCl_4 according to the method by Chang *et al*. with slight modifications[19]. In brief, K_2PtCl_4 was dissolved in ultrapure water to give a final concentration of 0.25 mM, and appropriate amounts of ascorbic acid and citric acid were simultaneously added into the solution while stirring at room temperature. Typically, Pt-NPs with a particle size of 30 nm were prepared by adding ascorbic acid and citric acid to give final concentrations of 2.5 mM and 0.25 mM, respectively. The solution was stirred for 5 min and left until the mixture became dark black (12 h) at room temperature. The mean particle size and zeta-potential were analyzed using a microparticle analyzer (Zetasizer Nano, Malvern Instruments Ltd., Worcestershire, UK) at 25°C. We defined Pt-NPs with a particle size of 106 nm as large and those with a particle size of 30 nm as small Pt-NPs. Zeta-potentials of large and small Pt-NPs were -3.4 and -23.3 mV, respectively. In addition, we observed that the prepared Pt-NPs were

spherical and had diameters generally ranging from 20 nm to 200 nm, on the transmission electron microscope (TEM) images (data not shown), which is consistent with the results of mean particle size obtained using a microparticle analyzer.

Scavenging of superoxide anion by platinum nanoparticles

The ability of Pt-NPs to scavenge superoxide anion was assessed according to a previously published method with slight modifications [20]. Briefly, large or small Pt-NPs were added to a 5% glucose solution containing 0.2 mM xanthine and 0.024 μunits xanthineoxidase to give an equivalent final platinum concentration of 0.024 μM. The mixture was stirred for 10 min at 37°C in the dark, and CLA was added to the solution to give a final concentration of 16 μM. The light produced was immediately measured with a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany).

Scavenging of hydrogen peroxide by platinum nanoparticles

The ability of Pt-NPs to scavenge hydrogen peroxide was assessed using a previously published method with slight modifications [21]. Briefly, large or small Pt-NPs were added to a 5% glucose solution containing 1000 μM hydrogen peroxide at various platinum concentrations. The mixture was stirred for 60 min at 37°C in the dark, and

BES-H_2O_2 was added to the solution to give a final concentration of 1.7 μM. The fluorescence intensity of these samples was measured by a spectrofluorometer (SpectrafluorPlus, TECAN, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Scavenging of hydroxyl radicals by platinum nanoparticles

The ability of Pt-NPs to scavenge hydroxyl radicals was assessed according to the method published by Setsukinai et al. with slight modifications [22]. In brief, the fluorescent hydroxyl radical probe HPF was dissolved in a 5% glucose solution containing 890 μM hydrogen peroxide to a final concentration of 3.0 μM. Large or small Pt-NPs were added to the solution at various platinum concentrations. The mixture was stirred for 10 min at room temperature under UV radiation immediately after the addition of Pt-NPs. The fluorescence intensity of these samples was measured as described above.

Tissue distribution of platinum nanoparticles in mice

Large or small Pt-NPs at a dose of 50 μg platinum/kg were injected into the tail vein of ddY mice. At predetermined times after injection, blood was collected from the vena

cava under ether anesthesia, and the mice were then killed. Heparin sulfate was used as an anticoagulant. Plasma was obtained from the blood by centrifugation. The liver, kidneys, spleen, heart, and lungs were removed, rinsed with saline, and weighed. The tissues were dried by lyophilization. Blood, plasma, or dried tissue was heated at 200°C with 60% HNO_3, 30% H_2O_2, and 60% $HClO_4$, respectively, in 50-mL beakers. The procedure was repeated until a white residue was obtained. The residue was dissolved and heated at 120°C with aqua regia. The resulting residue was dissolved in 10 mL of 7 % nitric acid. Platinum concentrations were determined with an inductively coupled plasma mass spectrometer (ICP-MS) (ICPM-8500, Shimadzu Co., Kyoto, Japan)[23].

Hepatic cellular localization

The separation of liver parenchymal cells and nonparenchymal cells was performed by using the collagenase perfusion method[24]. Briefly, mice were anesthetized with pentobarbital sodium (40–60 mg/kg) and given an intravenous injection of large or small Pt-NPs. A heat lamp kept the body temperature at 37°C during the experiment. Then, 30 min after Pt-NPs administration, the liver was perfused with a Ca^{2+}- and Mg^{2+}-free perfusion buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES], 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, and 0.4 mM Na_2HPO_4,

pH 7.2) for 10 min followed by a perfusion buffer supplemented with 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I; pH 7.5) for 10 min. After the perfusion started, the vena cava and aorta were cut immediately, and the perfusion rate was maintained at 2 ml/min. After completing the perfusion, we excised the liver and removed its capsular membranes. The cells were dispersed by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through cotton mesh sieves and centrifuged at 50 × *g* for 1 min. The pellets containing parenchymal cells (PC) were washed twice with Hank's-HEPES buffer by centrifuging at 50 × *g* for 1 min. The supernatant containing nonparenchymal cells (NPC) was similarly centrifuged twice. The resulting supernatant was then centrifuged twice at 200 × *g* for 2 min. The PCs and NPCs were resuspended separately in 2.0 ml of ice-cold Hank's-HEPES buffer. The cell numbers and viability were determined by using the trypan blue exclusion method. The platinum concentration in the cells was determined with an ICP-MS as described above.

Hepatic ischemia/reperfusion

Under ether anesthesia, an incision was made in the abdomen of mice, and the portal vein and the hepatic artery were occluded with a vascular clamp for 15 min to induce

hepatic ischemia[5,6,9]. Then, blood was allowed to reflow through the liver (reperfusion). Saline or large or small Pt-NPs at an equivalent platinum dose (50 μg platinum/kg) were given via the tail vein just before the start of reperfusion. After 6 h of reperfusion, blood was collected from the vena cava, and plasma was obtained by centrifugation. ALT and AST activities, indicators of hepatocyte injury, were assayed using commercial test kits (Transaminase CII-Test Wako, Wako Chemical, Osaka, Japan). The liver was excised, rinsed with saline, and minced. Lipid peroxide levels in the liver were determined by using a thiobarbituric acid (TBA) method as reported previously[25]. The ratio of oxidized GSH (GSSG) to reduced glutathione (GSH) in the liver was measured by using commercial test kits (GSSG/GSH Quantification Kit, Dojindo, Kumamoto, Japan). Control values were determined using livers obtained from age-matched, untreated mice.

Statistical analysis

The statistical comparisons were evaluated using an analysis of variance with subsequent Tukey's multiple comparison tests.

Results

Scavenging of ROS by platinum nanoparticles

Fig. 1 shows the effects of large and small Pt-NPs on the concentrations of superoxide anion, hydrogen peroxide, and hydroxyl radicals in 5% glucose solutions. The superoxide anion, hydrogen peroxide, and hydroxyl radical levels were significantly reduced in a concentration-dependent manner upon addition of large or small Pt-NPs. Furthermore, small Pt-NPs showed a greater reduction in levels for all three ROS in comparison to large Pt-NPs, although there was no significant difference in the decrease in hydrogen peroxide levels at a high platinum concentration (500×10^{-6}).

Distribution of platinum nanoparticles after intravenous injection in mice

Fig. 2 shows the time course for the distribution of large and small Pt-NPs in the plasma and major organs after intravenous injection into mice. Large and small Pt-NPs rapidly disappeared from the plasma. Approximately 80–100% of the platinum accumulated in the liver within 10 min of injection of large and small Pt-NPs. Although large Pt-NPs gradually accumulated in the spleen, there was no significant accumulation of platinum in the kidney, heart, lung, or urine. Fig. 3 shows the hepatic cellular localization of Pt-NPs 30 min after intravenous injection in mice. Large and small

Pt-NPs selectively accumulated in NPC with an NPC/PC ratio of 21.4 and 14.0, respectively.

Prevention of hepatic ischemia/reperfusion injury by platinum nanoparticles

Fig. 4 shows the activity levels of AST and ALT in plasma of hepatic ischemia/reperfusion-injured mice. The AST and ALT activities in plasma significantly increased 6 h after reperfusion from approximately 100 to 650 IU/ml and from approximately 15 to 290 IU/ml in untreated mice, respectively. The elevation in AST and ALT activity was effectively prevented by large or small Pt-NPs, although large Pt-NPs had no statistically significant effects on AST activity. Furthermore, small Pt-NPs were more effective than large Pt-NPs at reducing both AST and ALT activity levels.

Lipid peroxide content in the liver after ischemia/reperfusion

Fig. 5 shows the lipid peroxide content in the livers of untreated mice and mice treated with hepatic ischemia/reperfusion. After 6 h of reperfusion, the lipid peroxide content of the livers was 1.8 times higher than the levels before ischemia. Pt-NPs

effectively reduced the increase in lipid peroxide content, but this reduction reached statistical significance for only the small Pt-NPs.

The ratios of oxidized GSH to reduced GSH in the liver after ischemia/reperfusion

We measured the ratio of GSSG to reduced GSH in the liver after ischemia/reperfusion as an indicator of oxidative stress in this organ[26]. The ratio of GSSG to reduced GSH increased from a baseline level of 0.6 to 0.9 at 6 h of reperfusion. Although the increase in the ratio of GSSG to reduced GSH was suppressed by the administration of Pt-NPs, this suppression reached statistical significance for only the small Pt-NPs.

Discussion

Recently, various species of metal nanoparticles have been developed for drug delivery and medicine. Gold nanoparticles have been used as drug carriers[27,28]. Cerium and yttrium nanoparticles have been reported to effectively suppress cellular toxicity by scavenging ROS *in vitro*[29]. Pt-NPs have been widely used as a common ingredient in food since their approval as a food additive by the Ministry of Health, Labour and Welfare of Japan. Some researchers have reported that Pt-NPs have high

ROS-scavenging activities and effectively suppress ROS-mediated diseases[16,17]. However, little attention has been paid to tissue distribution, which is related to therapeutic effect and toxicity. To our knowledge, the results presented here constitute the first demonstration of the pharmacokinetics and efficacy of Pt-NPs to prevent hepatic ischemia/reperfusion injury.

As shown in Fig. 1, Pt-NPs effectively scavenged various types of ROS, and the scavenging activity of small Pt-NPs was higher than that of large Pt-NPs. This scavenging ability of Pt-NPs is likely due to the catalytic activity conferred by the high ratio of electrons remaining on the particle surface and therefore readily available to chemically transform substrate. Smaller particles have reportedly greater catalytic activity than do larger particles, due to the larger surface area of smaller particles[30,31]. Therefore, smaller Pt-NPs would have an advantage in scavenging ROS.

In hepatic ischemia/reperfusion, NADPH oxidase activation leads to the generation and accumulation in Kupffer cells of ROS, which, in turn, recruits and activates neutrophils. Once activated and attached to endothelial cells, neutrophils may exacerbate tissue injury by generating ROS and secreting several proteases, such as myeloperoxidase, elastase, and collagenase[32,33]. These results suggest that the delivery of ROS scavengers to liver nonparenchymal cells, such as Kupffer and sinusoidal

endothelial cells, is a promising approach for preventing hepatic ischemia/reperfusion injury[9]. It was reported that microparticles or nanoparticles are usually trapped by the reticuloendothelial system (RES), including macrophages (i.e., Kupffer cells) and macrophage precursors in the liver [18]. It was also reported that the uptake of particles by macrophages increases with increasing particle size [34]. In the present study, we demonstrated that Pt-NPs rapidly accumulated in the liver after intravenous injection and that the accumulation of large Pt-NPs was greater than that of small Pt-NPs (Fig. 2). Furthermore, these Pt-NPs predominantly accumulated in NPC, including Kupffer cells and endothelial cells (Fig. 3). These results are in good agreement with those from previous reports examining existing NPs [18,34]. Therefore, this greater hepatic clearance of Pt-NPs could be the result of uptake by Kupffer cells in the liver, affording a greater opportunity to scavenge ROS in the liver.

In the present study, ischemia followed by reperfusion resulted in a remarkable increase in plasma AST and ALT activities (Fig. 4), indicating severe liver damage due to treatment [5, 6]. However, this increase in AST and ALT levels was effectively reduced by Pt-NPs, indicating that Pt-NPs prevent hepatic injury after hepatic ischemia/reperfusion. Furthermore, the efficacy of small Pt-NPs was superior to that of large Pt-NPs, even though the hepatic accumulation of large Pt-NPs was greater than

that of small Pt-NPs. This may be attributable to the greater ROS scavenging activity of small Pt-NPs compared with large Pt-NPs. These results together with ROS scavenging activities and pharmacokinetic data indicated that the therapeutic benefits were influenced by the balance between ROS scavenging activities and the delivery of Pt-NPs to the sites where ROS were generated (i.e., Kupffer cells).

To elucidate the ROS scavenging activities of Pt-NPs in hepatic ischemia/reperfusion, we assessed the effect of Pt-NPs on oxidative stress in hepatic ischemia/reperfusion. It is well known that ROS induce membrane lipid peroxidation in ischemia/reperfusion[35]. In addition, GSH neutralizes ROS to form GSSG through a cascade of detoxification mechanisms[26]. Therefore, the lipid peroxide content and the ratio of GSSG to reduced GSH are frequently used as indicators of tissue response to ROS generation. We showed that in hepatic ischemia/reperfusion, large or small Pt-NPs reduced the increase in lipid peroxide content and the ratio of GSSG to reduce GSH in the liver in a manner similar to that observed for the results of AST and ALT activities (Fig. 5, 6), indicating that Pt-NPs suppressed the oxidative stress induced by ischemia/reperfusion. Rehman et al. reported that polyacrylic acid-protected Pt-NPs significantly reduced the LPS-induced production of intracellular ROS and inflammatory mediators in RAW 264.7 macrophages[36]. The transcriptional factor NF-κB plays a key role in

ischemia/reperfusion injury through regulation of a variety of gene expressions, such as cell adhesion molecules on both endothelial cells and neutrophils[37-39]. Thus, although further studies are needed to fully elucidate the detailed mechanisms whereby Pt-NPs prevent hepatic injury, considering the results of these previous studies together with our data suggests that Pt-NPs prevent oxidative stress and subsequently partly suppress inflammatory-related cellular events through prevention of NF-κB activation in the liver after ischemia/reperfusion.

Conclusion

We successfully developed Pt-NPs, which exhibited ROS scavenging activity and selective delivery to the liver. Pt-NPs effectively suppressed the hepatic injury in mice following hepatic ischemia/reperfusion. Although further long-term studies need to be performed to elucidate the elimination pathway and toxicities of Pt-NPs for clinical use, these findings indicate that Pt-NPs are promising compounds for the prevention of hepatic ischemia/reperfusion injury.

Acknowledgements

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Figure Legends

Fig. 1. Effects of Pt-NPs on superoxide anion (A), hydrogen peroxide (B) and hydroxyl radicals (C) in glucose solutions. The results are expressed as the mean ± S.D. of 3 samples. **$p < 0.01$, significantly different from the control group. †$p < 0.05$, ††$p < 0.01$, significantly different from the large Pt-NPs group.

Fig. 2. Plasma concentrations (% of dose/mL) and tissue accumulation (% of dose) of platinum after an intravenous injection of large Pt-NPs (A) and small Pt-NPs (B) at a dose of 50 μg of platinum/kg. ●, plasma; ▲, liver; ■, kidney; ○, spleen. The results are expressed as the mean ± S.D. for 3 mice.

Fig. 3. Hepatic cellular localization of Pt-NPs after intravenous injection into mice. Platinum concentrations were determined 30 min after injection of Pt-NPs. The results are expressed as the mean ± S.D. of 3 mice. Parenchymal cells, PC; Nonparenchymal cells, NPC

Fig. 4. Effects of Pt-NPs on plasma AST and ALT activity levels in mice with hepatic ischemia/reperfusion. Pt-NPs were administered at 50 μg of platinum/kg. The results are expressed as the mean ± S.D. of 3 to 7 mice. *$p < 0.05$, **$p < 0.01$, significantly different from the saline-treated group.

Fig. 5. The lipid peroxide content in mouse liver 6 h after hepatic ischemia/reperfusion. Lipid peroxide was detected by the thiobarbituric acid (TBA) method. Each of the Pt-NPs was administered at an equivalent platinum dose (50 μg of platinum/kg). The results are expressed as the mean ± S.D. of 3 to 7 mice. *$p < 0.05$, significantly different from the saline-treated group.

Fig. 6. Effect of Pt-NPs on the ratios of GSSG to reduced GSH in the livers of mice 6 h after reperfusion. Each of the Pt-NPs was administered at an equivalent platinum dose (50 μg of platinum/kg). The results are expressed as the mean ± S.D. of 3 to 7 mice. *$p < 0.05$, significantly different from the saline-treated group.

Fig. 1 (A)

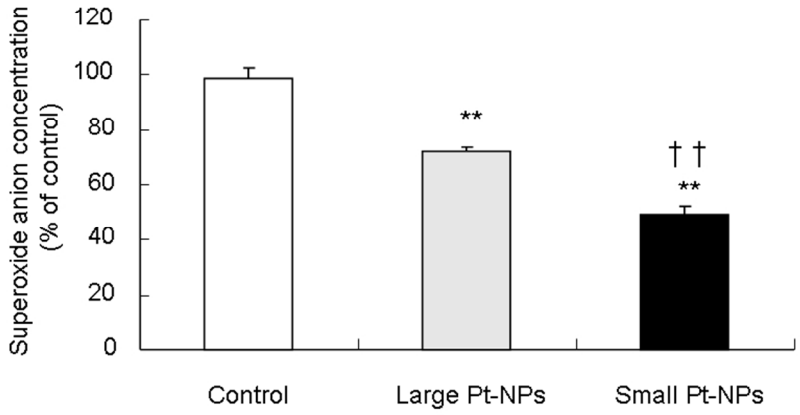

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Fig. 1 (B)

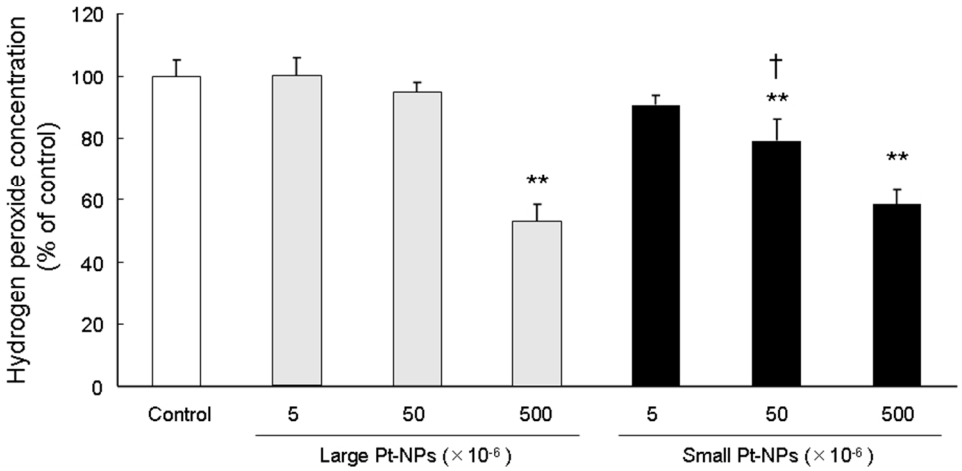

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Fig. 1 (C)

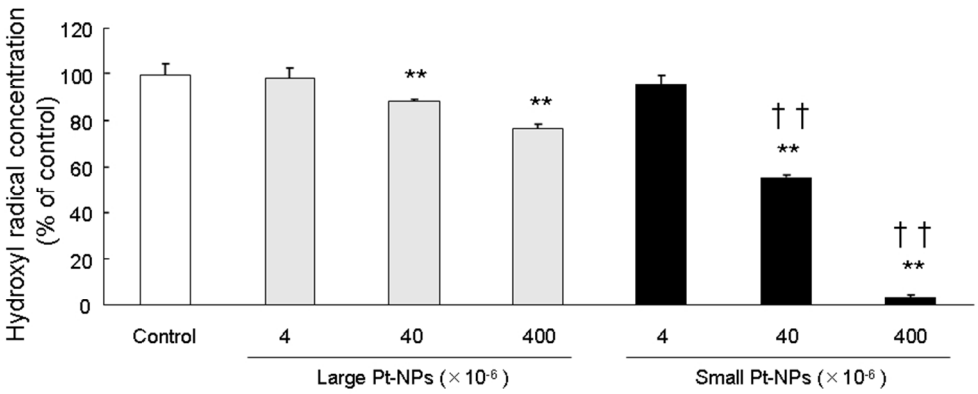

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Fig. 2 (A)

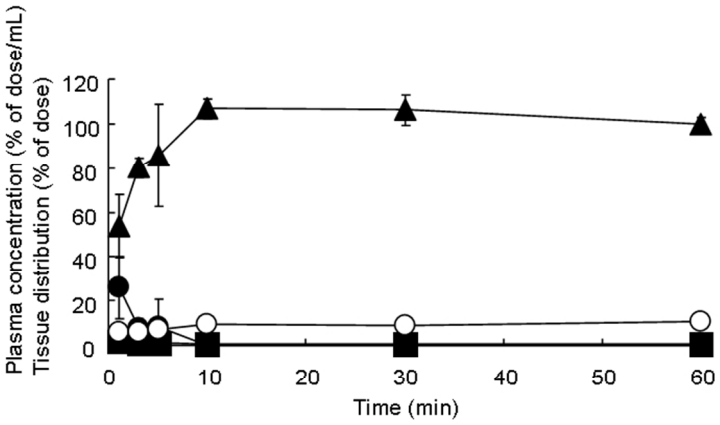

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Fig. 2 (B)

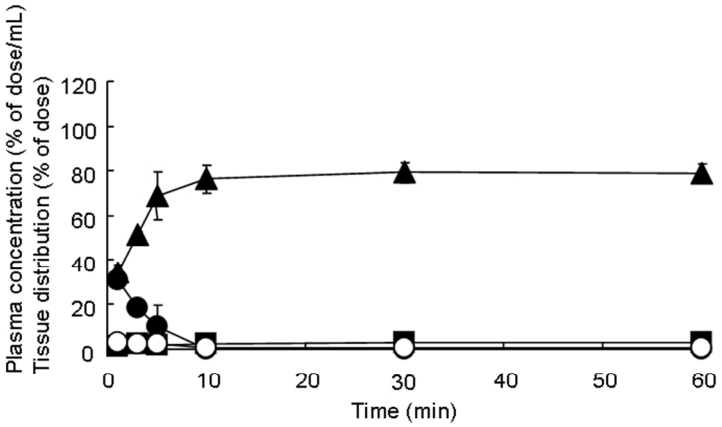

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Fig. 3

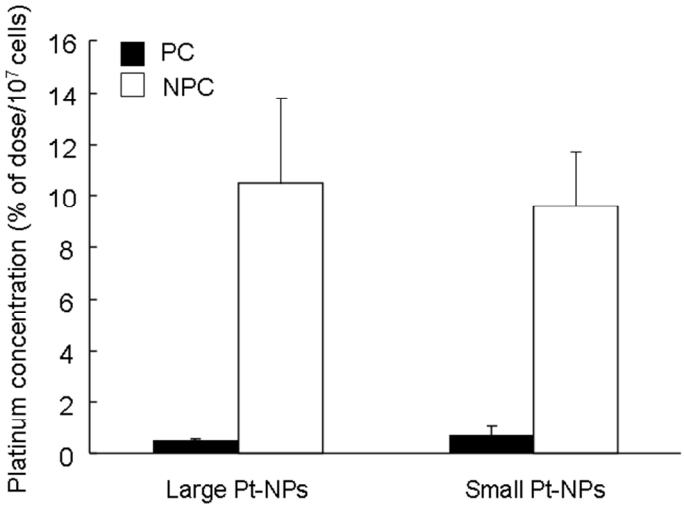

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Fig. 4

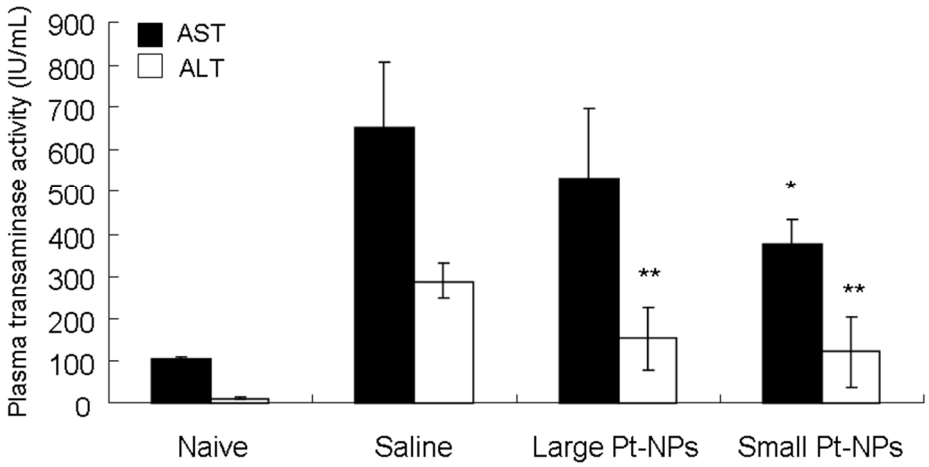

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Fig. 5

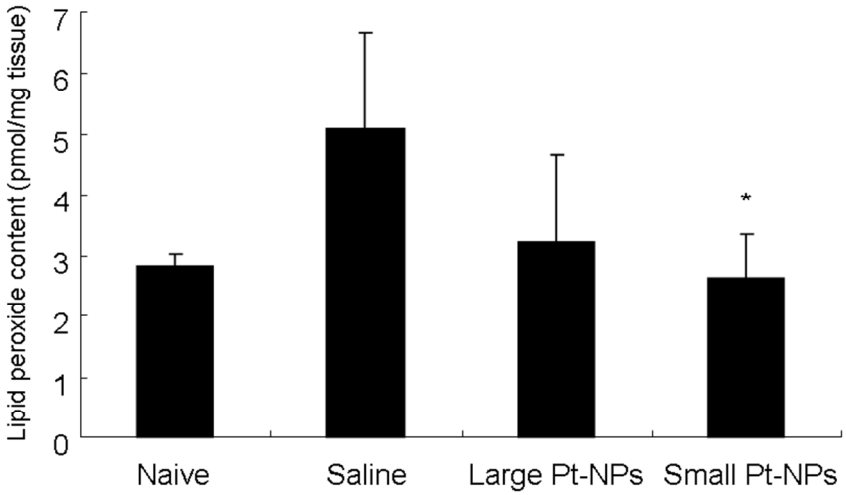

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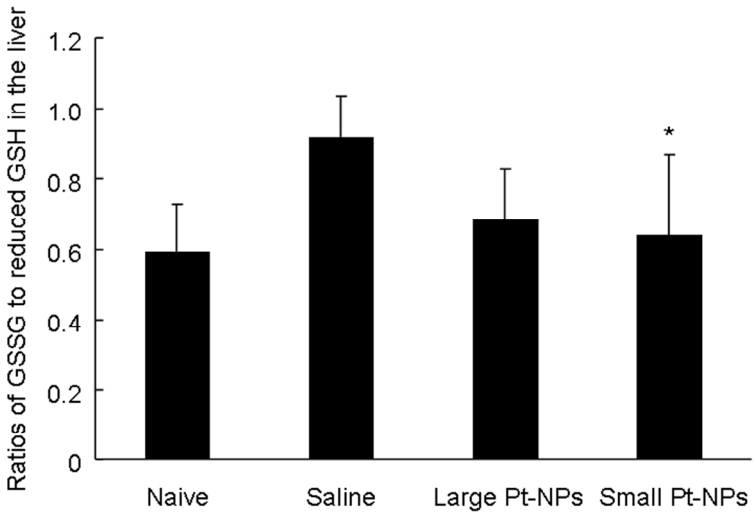
Fig. 6



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