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RESEARCH PAPER

Liposomes Coloaded with Iopamidol/Lipiodol as a RES-Targeted Contrast Agent for Computed Tomography Imaging

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ABSTRACT

Purpose The need for computed tomography (CT) of reticuloendothelial system (RES)-rich organs such as the liver is increasing, particularly in patients with suspected hepatic metastasis. CT images of the liver have been improved by encapsulating currently used, water-soluble iodine contrast agent in liposomes. The present study was performed to investigate a possibility to overcome the limitations of entrapped iodine in liposomes by preparing liposomes co-loaded with iopamidol, a water-soluble iodinated compound, and lipiodol, an iodized oil.

Methods Iopamidol and lipiodol were simultaneously loaded in liposomes by modified reverse-phase evaporation method. The entrapped iodine concentration, mean particle size and polydispersity index of resulting liposomes were evaluated. Following intravenous injection of these liposomes into rats, CT scanning was performed.

Results Simultaneous loading of iopamidol and lipiodol into liposomes resulted in entrapped iodine concentrations as high as 49.2 iodine mg/ml. The mean particle size was 280 nm, and the mean polydispersity index was 0.230. CT scanning with these iopamidol/lipiodol (I/L) liposomes into rats resulted in more pronounced and more persistent increases in RES-rich organs, liver and spleen, compared with free liposomes or liposomes loaded with iopamidol alone.

Conclusions These findings indicate that I/L liposomes have the potential to allow thorough CT examination of RES-rich organs.

KEY WORDS computed tomography · contrast agent · iopamidol · lipiodol · liposome

INTRODUCTION

Liposomes have been investigated extensively for use in therapy and diagnosis (1). The biocompatibility of phospholipids provides liposomes with pharmaceutical acceptability, especially for injectable formulations. Liposomes can carry a wide variety of materials, and their use as carriers has several advantages, including increased *in vivo* half-life of the entrapped substances. For example, liposomal encapsulation of amphotericin B not only increased its solubility, but also increased its *in vivo* half-life with reduced nephrotoxicity (2).

Computed tomography (CT) is currently used for the diagnosis of a wide range of diseases, including cancers. In CT, contrast agents are generally administered to increase visibility in the area of the body being studied (3). The need to image reticuloendothelial system (RES)-rich organs, such as the liver, is enormous, particularly in the preoperative evaluation of patients with suspected hepatic metastasis (4). In imaging RES-rich organs, however, currently used water-soluble contrast agents may be not ideal because their intravascular residence time is brief and they are rapidly excreted.

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One method of opacifying the liver on CT images is to encapsulate water-soluble contrast agents into particulate carriers. The particulate nature of liposomes leads to their rapid uptake by the RES, making intravenously administered liposomes particularly useful for carrying substances to RES-rich tissues (5). For example, several studies have demonstrated that water-soluble contrast agents entrapped in liposomes were taken to RES-rich organs and produced enhanced contrast of the liver and spleen in CT (6–8). A major drawback of previously described contrast material-carrying liposome preparations has been their limited encapsulation efficiency. Although relatively high liposome-entrapped iodine concentrations have been reported in a limited number of recent studies, these liposomes required excess concentrations of phospholipids and the use of a high volume of ethanol as a solvent, which was later removed only by dialysis (9).

Lipiodol, an iodinated ethyl ester of poppy seed oil, is clinically used as an embolizing agent (10) and as a contrast agent for lymphography (11). Because small amounts of oils can be loaded into liposomes by embedding these oils in the hydrophobic portions of liposome lipid bilayers (12), a possibility to investigate whether adding a small amount of lipiodol into phospholipid bilayers during liposome preparation could result in the production of liposomes with highly increased iodine concentrations was investigated in the present study. The present study describes the preparation of liposomes co-loaded with iopamidol, a water-soluble iodinated compound, and lipiodol, and show that these liposomes resulted in improved images of RES-rich organs of rats in CT, compared with free liposomes or liposome loaded with iopamidol alone.

MATERIAL AND METHODS

Materials

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-Dimyristoyl-sn-glycero-3-[Phospho-L-Serine] (DMPS) were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, USA). Cholesterol (Chol) and Stearylamine (SA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Iodized oil Lipiodol was purchased from Guerbet (Aulnay-Sous-Bois, France), and iopamidol was kindly provided by Accuzen (Seoul, Korea). All other chemicals were of reagent grade and used without further purification.

Preparation of Liposomes

Liposomes were prepared by modifying the previously published reverse phase evaporation (REV) method (13) with slight modifications. Briefly, 120 μmole of lipids were dissolved in tertiary butyl alcohol. When addition of Lipiodol was required, the appropriate amount of Lipiodol was also dissolved in tertiary butyl alcohol. After rapid freezing at −70°C, mixtures were subjected to freeze-drying by freeze dryer (EYELA FDU-1200, Japan). Finely dispersed lipid cakes obtained after overnight drying were redissolved in 13.5 ml of an organic phase consisting of isopropyl ether and chloroform in the volume ratio 2:1. Three milliliter of aqueous phase containing iopamidol dissolved in phosphate-buffered saline (PBS, pH 7.4) was added dropwise to the organic phase. The organic and aqueous phases were emulsified using a bath-type sonicator for 20 min at 37°C. The organic phase was then removed under slightly reduced pressure until a clear suspension was obtained. 1.5 ml of PBS was added again, and the residual organic phase was removed under greatly reduced pressure. The resulting liposome dispersions were dialyzed overnight to remove non-entrapped materials. When required, liposomes were concentrated using stirred ultrafiltration cells (molecular weight cut-off: 10 kD; Millipore Corporation, MA) and stored at −4°C until use.

Characterization of Liposomes

The mean particle size and polydispersity index (P.I.) of phospholipid dispersions were determined by dynamic light-scattering method using fiber-optics particle analyzer (FPAR-1000, Otsuka Electronics, Japan). Prior to measurement, dispersions were diluted with filtered saline. The system was used in the auto-measuring mode. The P.I. is a measure of the uniformity of the particle size distribution in a system studied (14).

Zeta potential of liposomes was determined by using the electrophoretic light-scattering spectrophotometer (ELS-8000, Photal, Osaka, Japan). Prior to determination, lipid dispersions were diluted until analytical measurement range in filtered saline (14). Data analysis was conducted using a software supplied by the manufacturer.

To determine the iodine concentration loaded in liposomes, serial dilutions of iopamidol solutions with known iodine concentrations were made, and their Hounsfield Unit (HU) values were obtained by CT scan to construct a standard curve. HU value of each liposome sample was obtained and converted to iodine concentration by using the standard curve.

Phospholipid concentration in lipid dispersions was determined by the Stewart assay (15).

In Vivo Study

Female, nine-week-old Crj/Bgi–SD rats weighing 350 to 450 g (Charles River Japan Inc., Japan) were used in all experiments. Rats were housed in a plastic cage (2–3 per

cage) with wood chip bedding, allowed access to food and water *ad libitum*, and maintained on a 12-hour dark: 12-hour light cycle in a temperature (23±2°C)- and humidity (55±10%)-controlled room. Rats were anesthetized with a mixture of Zoletil® (Virbac, Carros, France) (50 mg/kg) and xylazine (10 mg/kg) given by intraperitoneal injection. Prepared contrast materials were immersed in a 36.5°C water bath for 30 min before injection. After precontrast CT scan was performed, contrast materials were injected via distal tail vein. Re-anesthesia was performed when necessary for the delayed CT scan. All protocols used in the present studies were reviewed and approved by the Experimental Animal Ethical Committee of Yonsei University according to *The Guide for the Care and Use of Laboratory Animals*.

CT Image Acquisition and Analysis

At designated time points, CT images were obtained with a commercialized 16-channel multi-slice CT (Sensation 16; Siemens, Medical Systems, Forcheim, Germany) using the following parameters: rotation time 0.5 s, 120 kV, 310 mAs, beam collimation 0.6 mm, and slice thickness 1 mm. Images were obtained at pre-, 0, 1, 2, 3, 6, 10, 30, 90, 150, 210 min, 24, 64 h and 1 week. Determined regions were positioned in the liver, spleen, aorta and kidney.

Imaging data from the CT scans were transferred to a PACS workstation (Centricity, GE Medical Systems, Milwaukee, Wis) for quantitative evaluation. To assess the enhancement achieved with the contrast agents, the CT signal intensity was expressed in Hounsfield units (HU). All image measurements were obtained by the same experienced CT radiologist.

Statistical Analysis

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's t-tests.

RESULTS

Effect of Composition on the Iodine Concentration in Liposomes

To obtain liposomes with maximized iodine concentration, it was first examined whether liposome composition could affect the concentration of incorporated iopamidol. The effect of cholesterol incorporation on the incorporation efficiency was first investigated, since cholesterol is known to increase the rigidity of liposomal membranes (16). Liposomes prepared with a mixture of DMPC and cholesterol resulted in an entrapped iopamidol concentration 1.6-fold higher than that obtained with liposomes prepared with DMPC alone (Fig. 1A), indicating that the increased liposome rigidity due to cholesterol inhibited the leakage of entrapped iopamidol during the liposome preparation process.

Electrostatic repulsion between charged lipids in the liposome membrane can increase liposome size, thereby increasing the volume of the inner aqueous core in which water-soluble materials can be entrapped. When liposomes differing in surface charges were prepared, it was found that the zeta potential of liposomes prepared with DMPC/cholesterol was slightly negative (−18 mV). In contrast, the zeta potential of liposomes prepared with DMPC/cholesterol/SA was highly positive (+46 mV), whereas the zeta potential of liposomes prepared with DMPC/cholesterol/DMPS was highly negative (−88 mV). However, the entrapped iodine concentration was not increased by altering the surface charge of liposome membranes; rather, the iodine concentration was decreased in highly charged liposomes, particularly in negatively charged liposomes (Fig. 1B).

The highest iodine concentration that could be obtained by loading iopamidol into DMPC/cholesterol liposomes was approximately 14 mg/ml. To further increase the concentration of entrapped iodine, liposomes were prepared with a combination of iopamidol and lipiodol, an iodized oil. Addition of 25, 50 or 75 μl lipiodol to a 120 μmole mixture of DMPC/cholesterol during the initial step of liposome preparation resulted in homogeneous lipid dispersions after reverse-phase evaporation, with a concentration of entrapped iodine proportional to the amount of lipiodol added (Fig. 1C). Addition of more than 75 μl lipiodol resulted in a mixture too viscous after reverse-phase evaporation, thereby failing to produce liposomal dispersion. The iodine concentration entrapped in liposomes prepared with both iopamidol and lipiodol (49.2 iodine mg/ml) corresponds to the approximate sum of the iodine concentrations entrapped in liposomes prepared with iopamidol alone (13.8 iodine mg/ml) and lipiodol alone (32.7 iodine mg/ml), indicating that the addition of up to 75 μl lipiodol did not decrease the inner aqueous volume of liposomes. Therefore, it is likely that 120 μmoles of lipids could contain up to 75 μl lipiodol to form liposome layers.

Effect of Lipiodol Addition on the Physical Properties of Liposomes

To investigate whether the addition of lipiodol changed the physical properties of liposomes, the mean particle size and polydispersity index (PI) of liposomes were measured in relation to the amount of incorporated lipiodol. Incorpo-

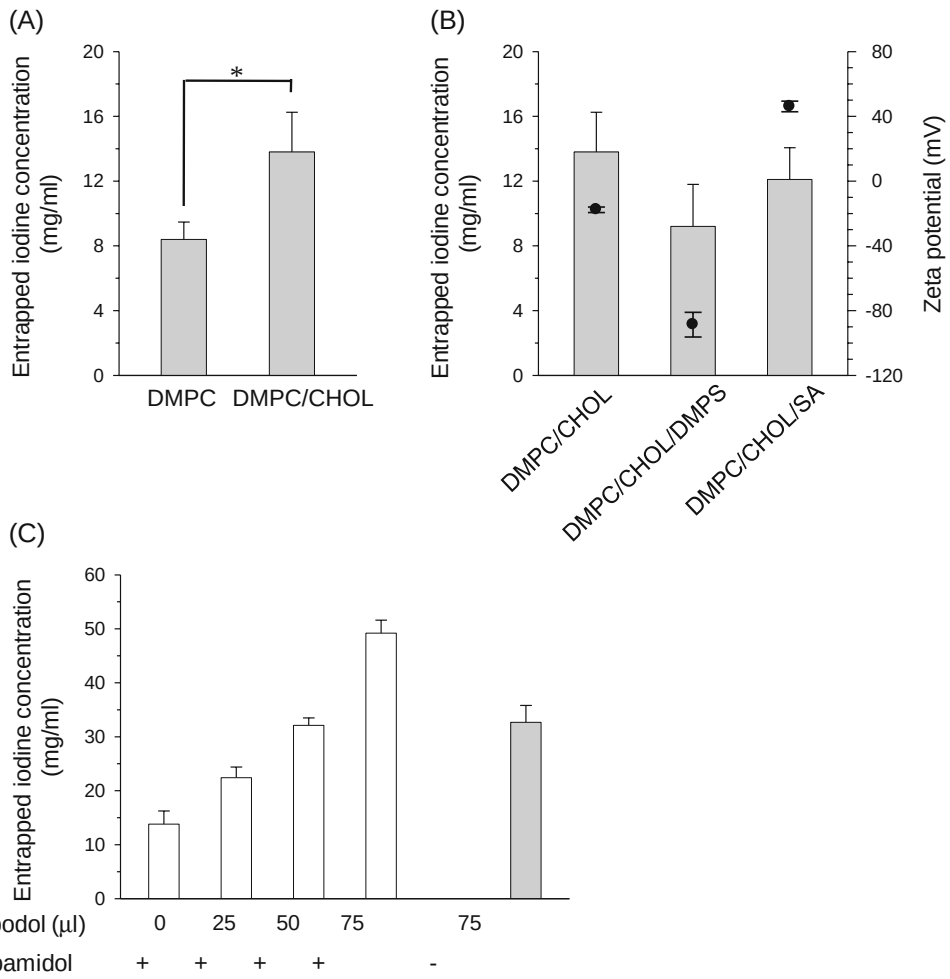

Fig. 1 Effect of **A** cholesterol addition, **B** surface charge and **C** lipiodol addition on the iodine concentration loaded in liposomes. *Bars* represent the iodine concentration loaded in liposomes, and the points represent the zeta potential of liposomes. **A** DMPC alone or 3:2 (molar ratio) mixture of DMPC:cholesterol (total 120 μmole) was used to prepare liposomes. Significant differences are indicated by asterisks: *, $P < 0.05$. **B** DMPC:cholesterol (24:16), DMPC:cholesterol:DMPS (24:10:6) or DMPC:cholesterol:SA (24:10:6) mixture was used to prepare liposomes. **C** Liposomes were prepared with 24:16 mixture of DMPC:cholesterol (total 120 μmole) with or without addition of indicated volume of lipiodol. As a control, liposomes were prepared in the presence of lipiodol alone (without iopamidol). Data are presented as means ± S.D. ($n = 3$).

ration of up to 75 μl lipiodol increased the mean particle size by 1.4- to 1.6-fold, compared with liposomes without lipiodol. PI was less than 0.3 regardless of lipiodol addition, indicating that homogeneity was maintained (Fig. 2A). Furthermore, the mean particle size of liposomes, with or without incorporated lipiodol, remained relatively unchanged for up to 4 weeks (Fig. 2B), suggesting that incorporation of up to 75 μl lipiodol did not destabilize these liposomes.

In Vivo Study

To determine whether liposomes containing lipiodol and iopamidol (I/L liposomes) resulted in better CT images of RES-rich organs, these liposomes, as well as liposomes prepared with iopamidol alone (I-liposomes) and free iopamidol solution, were intravenously injected into rats. The contrast enhancement characteristics of each contrast agent in specific organs are illustrated in the time-enhancement curves in Fig. 3. Liver enhancement in the rat started immediately after injection of I/L-liposomes; at 6 min post-injection, the enhancement reached clinically relevant levels (over 30 HU difference), which persisted for up to 24 h. In contrast, liver enhancement following the injection of free iopamidol solution, at the same iodine concentration as in I/L-liposomes, was minimal (<7 HU difference) throughout the entire observation period. I-liposomes containing a relatively low iodine concentration (14 mg/ml) increased liver enhancement compared with free iopamidol but remained low, never reaching an ΔHU higher than 20. The maximal liver CT enhancement obtained by I/L-liposomes was 8.3-fold higher than that obtained by free iopamidol solution (50 *vs* 6 ΔHU).

Rats receiving I/L- or I-liposomes showed spleen enhancement immediately after administration, reaching a maximum at 90 min and persisting for up to 62 h in both groups, followed by a gradual decrease to near baseline within 1 week. In contrast, no enhancement was observed after injection of free iopamidol solution. When I/L- and I-liposomes were compared, rats receiving I/L liposomes demonstrated greater contrast enhancement of the spleen than rats receiving I-liposomes (maximal HU enhancement of 684 and 298, respectively).

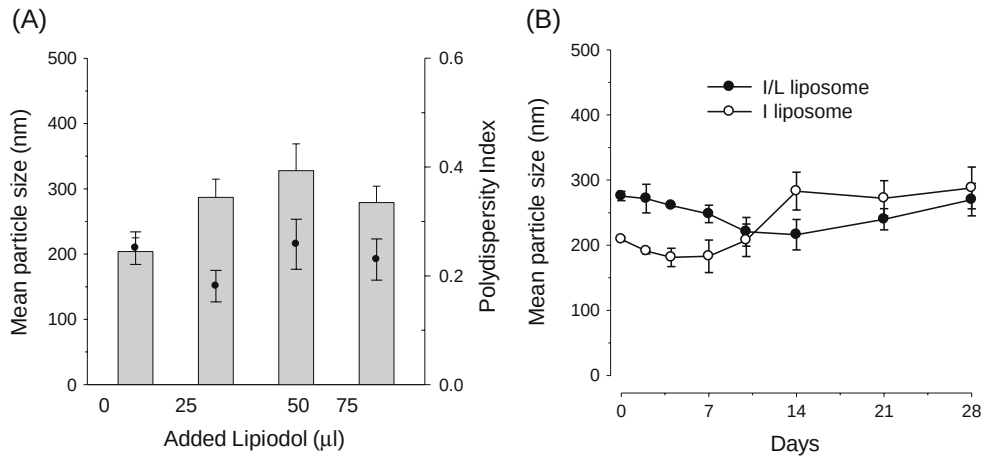

Fig. 2 Physical characteristics of iodine-loaded liposomes. **A** Effect of lipiodol addition on the mean particle size (□) and polydispersity (●) of liposomes. Liposomes were prepared with 24:16 mixture of DMPC:cholesterol (total 120 μmole) with or without addition of indicated volume of Lipiodol. **B** Effect of lipiodol addition on the stability of liposomes. Liposomes were prepared with 24:16 mixture of DMPC:cholesterol with or without addition of lipiodol (75 μl/120 μmole of lipids). The stability of liposomes was determined by monitoring the change in the mean particle size of liposomes during storage at 4°C. Data are presented as means ± S.D. ($n=3$).

Aorta enhancement was also observed immediately after administering I/L- and I-liposomes, indicating a high blood iodine concentration. The maximum increase in HU (94 *vs* 46 ΔHU) was 2-fold higher for I/L than for I-liposomes, and ΔHU was more than 30 for longer (there was a 15-fold difference; 150 *vs* 10 min). For both liposomes, CT values in the aorta gradually declined over time, decreasing to near basal level at 62-h postinjection. Compared with free iopamidol, I/L liposomes provided a 10-fold increase in the aorta over a 10-min period and a 3-fold increase over 2.5 h. Administration of free iopamidol solution did not result in significant HU enhancement, even during the immediate phase. Because the time required to obtain an immediate CT image is generally longer than 10 s, free iopamidol solution was already likely cleared from the blood at this time.

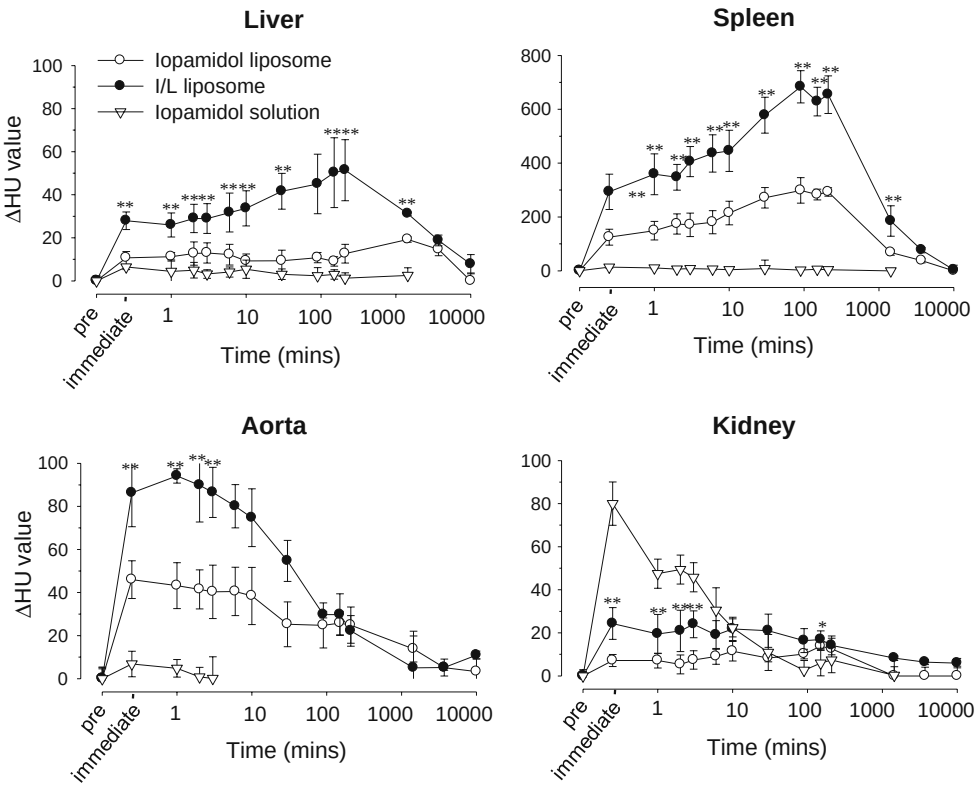

Fig. 3 Contrast enhancement curve as a function of time in **A** liver **B** spleen **C** aorta and **D** kidney obtained after intravenous administration of each contrast agent 190 mg I/kg for ioamidol/lipiodol liposomes (I/L liposome) and iopamidol solution, 108 mg/kg for iopamidol liposomes (I liposome), via distal tail vein of rat. Each point represents mean ± S.D. of at least three animals. Increase in HU for each time point the difference between the dynamic value and the initial value. Statistically significant differences compared with free iopamidol solution (*single asterisk*, $P<0.05$; *double asterisk*, $P<0.01$).

Free iopamidol showed an enhancement peak in the kidneys immediately after injection, followed by a rapid and steep decrease to near basal levels at 100-min post-injection. Both liposomes, however, showed little observable renal contrast enhancement. The initial renal peak observed with free iopamidol did not appear following liposome injection, and a moderate or slight increase in renal HU (≅20 ΔHU in I/L and ≅10 ΔHU in I-liposomes) was maintained from the immediate phase for up to 3.5–24 h.

Based on contrast enhancement curves (Fig. 3), representative CT images of each organ at the peak time point were obtained (Fig. 4). Opacification of the liver and spleen was most clearly observed with I/L liposomes, followed by I-liposomes, with the least clear observed with free iopamidol solution. Similarly, compared with I-liposomes, I/L liposomes provided increased image contrast between the aorta and the tissues surrounding the aorta. No detectable increases in the aorta images were observed with free iopamidol solution

DISCUSSION

Encapsulation of water-soluble iodinated contrast agents into particulate carriers such as liposomes is an effective strategy to target the liver for iodine uptake. To overcome previous limitations in entrapped iodine concentrations, a possibility was investigated in the present study whether liposomes containing increased iodine concentrations could be prepared by co-loading water-soluble iodinated compounds together with a small amount of iodized oil. It was found that the entrapped iodine concentration could be maximized by co-loading iopamidol and lipiodol. The inclusion of lipiodol, which is probably located within the hydrophobic portions of the phospholipid bilayer, did not cause significant destabilization of liposomal structure, as shown by mean particle size and polydispersity index data. CT scanning following intravenous injection of these I/L-liposomes into rats produced more pronounced and more persistent increases in RES-rich organs, such as the liver and spleen. These findings indicate that I/L liposomes have the potential to allow thorough CT examinations of RES-rich organs by providing a larger imaging window, over 24 h.

Although other iodinated contrast agent-loaded liposomes have been developed in a number of earlier studies, direct comparisons of their entrapped iodine concentrations and RES-targetability were not feasible, owing to differences in the type of iodinated contrast agent, the methods of preparation, including the process used to separate free, unentrapped contrast agent from liposomes, and the type and resolution of CT (whole-body or micro-CT). For example, iodixanol (9,17), iopromide (7,18,19), diatrizoate (20) and iomeprol (8) have been loaded into liposomes and used with (6,9) or without (7,8,17–19) separation of free agents.

Iopamidol was chosen in the present study because it is currently a widely used contrast agent due to its improved safety and tolerability compared with conventional iodinated contrast agents (21) and because, to the best of our knowledge, its incorporation into liposomes has not previously been reported. Reverse-phase evaporation method was employed since it has been known to be able to trap large amounts of water-soluble materials. Despite the expected high encapsulation volumes of these liposomes, those obtained were loaded with very limited amounts of iopamidol, much lower than obtained previously (9,17). This may be related to the physicochemical nature of iopamidol, for example, its relative ease in leaking out of liposomes during preparation, compared with other iodinated compounds. However, the concentration of entrapped iodine could be maximized by adding lipiodol during liposome preparation. Furthermore, the REV method, even in the presence of lipiodol, resulted in liposomes with a very low PI (≅0.220), which is a desirable characteristic for liposomes not to cause embolism after intravenous administration. The physical stability of I/L-liposomes was demonstrated by the lack of change in mean particle size and PI during storage for 1 month, suggesting that I/L liposomes have the potential for clinical use.

Using I/L liposomes, it was found that spleen enhancement was much higher than liver enhancement, indicating greater splenic accumulation. Similar results have been reported in other rat studies (5–7,19), suggesting that this property may be species-specific. Moreover, maximum HU enhancement of the aorta (94 ΔHU) was observed upon injection of I/L liposomes, persisting for up to 10 min, followed by a gradual decrease over 24 h. The increase in aorta HU to more than 30, maintained for up to 2.5 h, indicates that these liposomes may also allow CT image acquisition of the blood. Previous studies have shown little aortic enhancement, despite liposomal encapsulation, regardless of observation time, which may be due to the rapid uptake of liposomal agents by RES systems. Maximum hepatic and splenic enhancement was observed after 210 and 90 min, respectively, indicating that RES uptake of I/L- liposomes was slower. Liposome particle size is known as a major determinant of RES uptake, with larger liposomes generally taken up by RES-rich organs more rapidly. Earlier studies, however, used particulate contrast agents with smaller sizes (100–150 nm) as blood pool-targeted CT agents (22,23) and those of larger sizes (>500 nm) as RES-targeted agents (6,19). Therefore, although the liposome pharmacokinetics were not evaluated, it seems that smaller-sized I/L liposomes (≅280 nm)

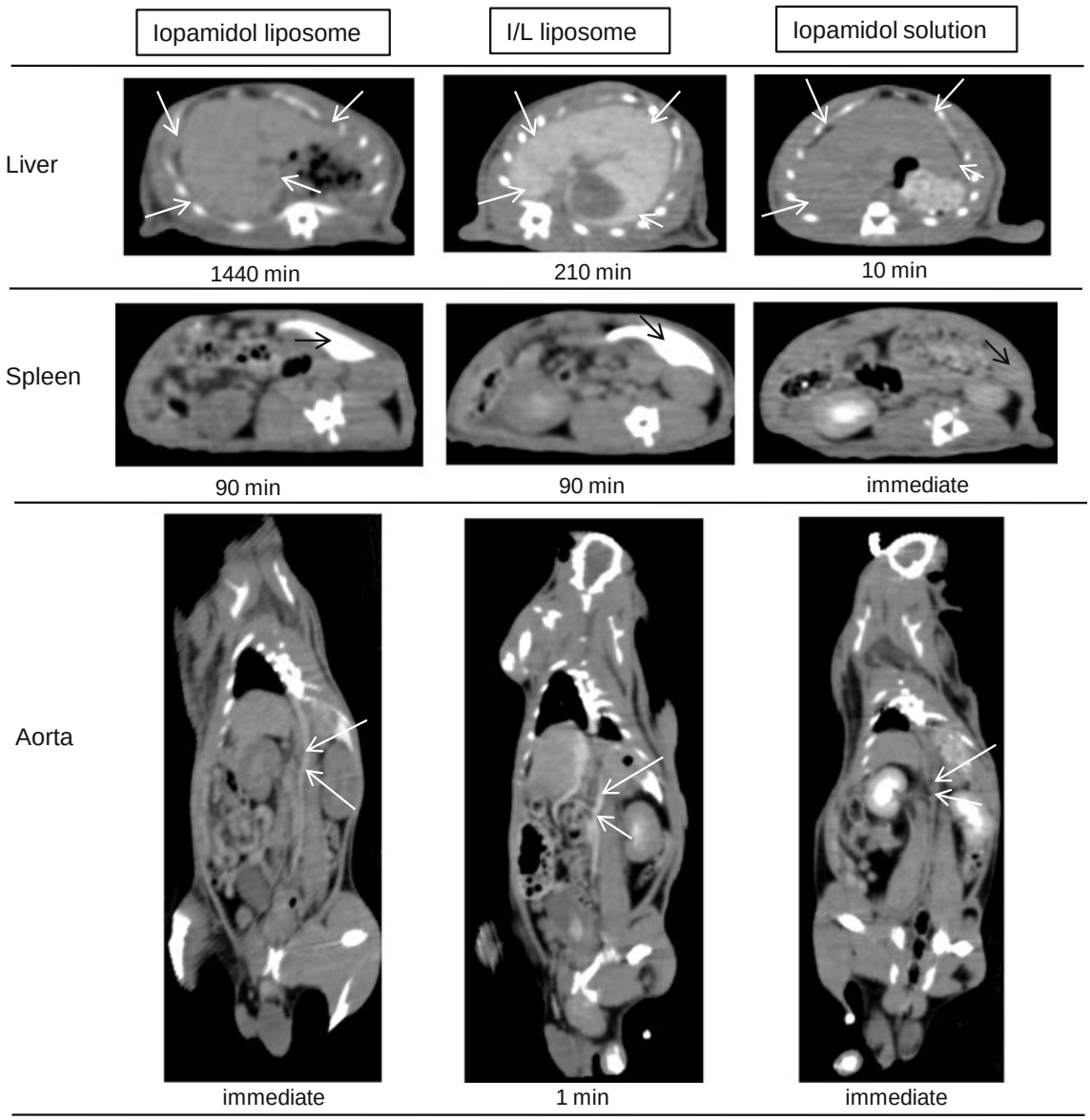

Fig. 4 Representative CT images of liver, spleen and aorta at designated time points following intravenous administration of each contrast agent via distal tail vein of rat. Each image was obtained at the time points showing the peak HU value.

were more slowly taken up by the RES system than the 2.9-μm liposomes previously described, thereby allowing blood pool imaging.

Most iopamidol is excreted in the urine (24). The disappearance of the renal enhancement peak observed after liposomal encapsulation showed that the usual mechanism used to clear the iodinated agent was altered by encapsulation. The liposomal agents were mainly cleared via the RES system, consistent with the known mechanism of clearance for liposomes. The potential renal toxicity of iodinated contrast agents in individuals with renal insufficiency (25) suggests that I/L liposomes may offer a clinical advantage by reducing the renal toxicity of contrast agents.

The dispersion of I/L liposomes described herein suggests that they have many advantages as RES-targeted contrast agents. These liposomes are easy to prepare, stable and provide pronounced and prolonged images of RES-rich organs. Due to the increasing clinical need for improved detection of liver tumors, detailed toxicological studies of I/L liposomes are needed prior to their use in other species including humans.

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