Grey scale enhancement of rabbit liver and kidney by intravenous injection of a new lipid-coated ultrasound contrast agent

Ping Liu, Yun-Hua Gao, Kai-Bin Tan, Zheng Liu, Song Zuo

INTRODUCTION

New ultrasound contrast consisting of perfluorocarbon microbubbles could enhance grey scale images of parenchymal organs. It provided a new method in diagnosis[1-10]. The development of new ultrasound contrast agent (UCA) has become one of the most promising fields in ultrasound medicine. So far, several UCAs, like Optison[11,12] and Definity[13], have been approved for treatment and become commercially available; many others are still in clinical trials[14-16]. Based on the chemical components of bubble film, UCAs could be divided into four different types: Surfactants[17-19], human albumin[20,21], polymer[22] and lipids[23-25]. The lipids are superior to the others in many aspects: no risk of blood transmitted infection, excellent stability and some tissue-specific targeting, like the reticuloendothelial system. This study was aimed to investigate the effectiveness, reproducibility of a new lipid contrast agent in the enhancement of abdominal parenchymal organs. All the protocols were approved by IRB (Internal Review Board) of Xinqiao Hospital.

METHODS:

Preparation and analysis of lipid-coated microbubble agent

Liposome, which consists of two kinds of phospholipids and polyethylene glycol, was prepared by lyophilization. Then the lipids were rehydrated with certain media, like glucose and deionized water. The resultant suspension was sonicated by an ultrasound sonicator (YJ 92-II Xinzhi Corp. Hang-zhou, Zhejiang Province). The perfluoropropane was introduced into the suspension during sonication, then it was kept still, until it was separated into two layers, the upper layer was still in white colloid whereas the lower layer was slowly defecated. Then the upper layer of the agent was extracted and analyzed. To acquire size distribution data of the new contrast agent, the milky microbubbles suspension was analyzed by Sysmex KX-21 (Sysmex Corporation, Japan). The surface electric potential and pH of UCA were measured by Zeta 3000 (Malvern Ltd., United Kingdom).

Animal models

Ten healthy rabbits, weighing 2.0-2.2 kg, were enrolled in this study. The body hair at experimental region was removed for liver and renal scan. Rabbits were anesthetized by intramuscular injection of “Xu Mian Xing” (mainly consist of haloperidol and made by Changchun University of Agriculture and Prologue) at the dose of 0.15 mL/kg bm, and the intravenous infusion was set up through ear veins. Lipid contrast agent was injected at 0.01 mL/kg bm, and followed by 1 mL saline flush.

Settings of ultrasound equipment

A Siemens Sequoia 512 (Siemens Acuson Co., Mountain View, California) ultrasound system was used in this study. The second harmonic imaging of 6L3 probe at 3.0/6.0 MHz was used. The mechanic index and output power were set at 0.11 and -24 dB, respectively. All the other parameters, like gain, depth, TGC, compress and focus, were kept constant during experiment. Experimental images were digitally recorded. Overall, baseline and 60 min contrast images were acquired. All images were transformed from original DICOM files into JPEG format by Viewpro (Acuson Co.). Grey scale was calculated by histogram in Adobe Photoshop 6.0 (Adobe Co.). Sample area used in the histogram had 1 088 pixels in an ellipse. Time-density curve was generated based on the mean pixel grey scale of hepatic parenchyma and renal cortex.

Pathological examination

All animals were sacrificed by intravenous injection of 10% potassium chloride. The liver, kidney and lung tissues were autopsied and fixed in 40 g/L formaldehyde for H&E stain. All tissue slides were reviewed by pathologists.

Statistical analysis

The grey scale data acquired from liver, inferior vena cava and...
renal cortex were expressed as mean±SD. Pre- and post-contrast of liver’s and kidney’s grey scale data were compared by paired two-tailed Student’s t test in SPSS 8.0 program. P critical value less than 0.05 was considered to be statistically significant.

RESULTS

Contrast agent
The visual appearance of the newly made liposome contrast agent was an opaque milky suspension, and it was slowly delaminated. The microbubble concentration was (7-8)×10⁹/mL, and the size distribution was 2 to 10 µm. About 90% of the microbubbles were less than 8 µm (Figure 1). The surface electric potential was -71.2 mV and the pH was 6.42.

Figure 1 Microbubbles in 400-fold diluted saline (original magnification: ×100).

Contrast agent

Grey scale enhancement of liver and kidney
The images of both hepatic parenchyma and renal cortex were visually significantly enhanced after intravenous injection of the contrast agent (Figures 2-5). Time-intensity curves of both liver and cortex were elevated and kept at a high level. The grey scale of liver parenchyma (61.2±3.1) 30 s after contrast injection was significantly higher than that of baseline image (38.0±3.0, $P<0.01$). And 54 min after the injection, the liver enhancement remained at 57.8±1.4. One hour after contrast injection, the grey scale dropped to 45.2±1.0, which had no statistical significance. Similar results were observed in the contrast enhancement of renal cortex, and the grey scales were 18.1±3.8 at baseline, 48.8±3.3 ($P<0.01$) 10 s after injection, 27.4±4.1 ($P<0.01$) 56 min after injection, 26.1±3.9 60 min after injection ($P<0.05$). These results confirmed that this lipid microbubbles contrast agent could effectively enhance hepatic parenchyma and renal cortex at a relatively long time.

Figure 3 Ultrasound images of liver parenchyma and inferior vena cava (IVC) before and after the injection of microbubbles. A: 15 min after injection; B: 25 min after injection; C: 30 min after the injection; D: 40 min after the injection.

Figure 4 Ultrasound images of renal cortex before and after the injection of microbubbles. A: before injection; B: 30 s after injection; C: 1 min after injection; D: 2 min after injection.
According to some reports\cite{27,28} and our experiment\cite{29}, wash-in and wash-out enhancement of microbubbles tended to be stable in parenchyma, rather than in cortex were quite different from that of inferior vena cava. These also, the time-intensity curves of hepatic parenchyma and renal cortex than some previous reports, which were about 5-15 min\cite{19,25,26}. The minimal dose of 0.01 mL/kg bm. Grey scale enhancement could the enhancement of hepatic parenchyma and renal cortex at a long-term stable in enhancement of liver parenchyma and renal cortex. The mean size of the microbubbles was less than 8 µm, which was smaller than that of red blood cell, and thereby it was safe for intravenous injection. Pathologically, all the tissues from liver, lung, and kidney were normal in histology, and there were no signs of air embolism or infarction spot.

In comparison with other lipid-coated microbubbles\cite{30}, this contrast agent had a higher bubble concentration and a longer stability in parenchymal organs. The reason for this might attribute to the reconstituted membrane of the microbubbles. Polyethylene glycol could protect phagocytosis from Kupffer cell, and it could exist in hepatic sinusoid longer. Tween 80, a kind of surfactant, which has never been used in other lipid microbubbles contrast agent, may increase the bubbles or microbubbles concentration.

Although this new lipid-based contrast agent exhibited a prolonged enhancement, the true reason has not been clarified. Further studies are needed to investigate the mechanism of long-lasting enhancement.

**DISCUSSION**

Lipid-coated microbubbles were mainly composed of several biocompatible phospholipids that had excellent stability and non-bioactive components. The lipids had been widely used in the preparation of ultrasound contrast agent, liposome and other drugs. In this study, a new ultrasound contrast agent based on lipids was prepared and showed excellent stability in the enhancement of hepatic parenchyma and renal cortex at a minimal dose of 0.01 mL/kg bm. Grey scale enhancement could last more than 50 min from both visual assessment and quantitative statistical analysis. This enhancement was longer than some previous reports, which were about 5-15 min\cite{19,25,26}. Also, the time-intensity curves of hepatic parenchyma and renal cortex were quite different from that of inferior vena cava. These microbubbles tended to be stable in parenchyma, rather than in circulation, as the time-density curve from IVC showed a 200-s wash-in and wash-out enhancement.

According to some reports\cite{27,28} and our experiment\cite{29}, we assumed that the prolonged enhancement was due to the uptake of microbubbles by the reticuloendothelial system in liver. But this could not explain the prolonged enhancement in the kidney, which is not rich of reticuloendothelial system. There might be some other reasons. Since the peak contrast intensities of liver parenchyma and cortex appeared much later than that of inferior vena cava, they were 12 min, 6 min and 30 s, respectively, it indicated that after the peak contrast in circulation there was another peak contrast in solid organs, and it was presumed to be the accumulation of microbubbles in hepatic sinusoids or capillary bed in cortex. As we know, hepatic parenchyma is consisted of hepatic sinusoids and renal cortex is mostly consisted of glomeruli. They are both rich in capillaries. Meanwhile, the blood flow in hepatic sinusoids and glomeruli is much slower than that in inferior vena cava. So, the microbubbles could easily be accumulated in capillary bed, which resulted in the prolonged enhancement of liver and kidney.

As IVC grey scale at 27 min postinjection was lower than that before injection, the hepatic parenchyma might block ultrasound transmission to IVC by condensed bubble’s attenuation, and this could produce visually lower IVC echo. In this study, the new lipids ultrasound contrast agent was long-term stable in enhancement of liver parenchyma and renal cortex. We assumed that the prolonged enhancement was due to the uptake of microbubbles by the reticuloendothelial system in liver. But this could not explain the prolonged enhancement in the kidney, which is not rich of reticuloendothelial system. There might be some other reasons. Since the peak contrast intensities of liver parenchyma and cortex appeared much later than that of inferior vena cava, they were 12 min, 6 min and 30 s, respectively, it indicated that after the peak contrast in circulation there was another peak contrast in solid organs, and it was presumed to be the accumulation of microbubbles in hepatic sinusoids or capillary bed in cortex. As we know, hepatic parenchyma is consisted of hepatic sinusoids and renal cortex is mostly consisted of glomeruli. They are both rich in capillaries. Meanwhile, the blood flow in hepatic sinusoids and glomeruli is much slower than that in inferior vena cava. So, the microbubbles could easily be accumulated in capillary bed, which resulted in the prolonged enhancement of liver and kidney.

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**REFERENCES**

1. Lindner JR, Song J, Christiansen J, Kilbanow AL, Xu F, Ley K. Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-Selectin. *Circulation* 2001; 104: 2107-2112
2. Bang N, Nielsen MB, Rasmussen AN, Osterhammel PA, Pedersen JF. Hepatic vein transit time of an ultrasound contrast agent: simplified procedure using pulse inversion imaging. *Br J Radiol* 2001; 74: 752-755
3. Rammarine KV, Kyriakopoulou K, Gordon P, McDicken NW, McArdle CS, Leen E. Improved characterisation of focal liver tumours: dynamic power Doppler imaging using NC100100 echo-enhancer. *Eur J Ultrasound* 2000; 11: 95-104
4. Marelli C. Preliminary clinical experience in cardiology with sonazoid. *Am J Cardiol* 2000; 86(Suppl): 10G-13G
5. Albrecht T, Blomley MJ, Cosgrove DO, Taylor-Robinson SD, Jayaram V, Eckersley R, Urbank A, Butler-Barnes J, Patel N. Non-invasive diagnosis of hepatic cirrhosis by transit-time analysis of an ultrasound contrast agent. *Lancet* 1999; 353: 1579-1583
6. Du WH, Yang WX, Wang X, Xiong XQ, Zhou Y, Li T. Vascularly of hepatic VX2 tumors of rabbits: Assessment with conventional power Doppler US and contrast enhanced harmonic power Doppler US. *World J Gastroenterol* 2003; 9: 258-261
7. Lindner JR. Detection of inflamed plaques with contrast ultrasound. *Am J Cardiol* 2002; 90(Suppl): 32L-35L
8. Wang WP, Ding H, Qi Q, Mao F, Xu ZZ, Kudo M. Characterization of focal hepatic lesions with contrast-enhanced C-cube gray scale ultrasonography. *World J Gastroenterol* 2003; 9: 1667-1674
9. Eyding J, Wilkening W, Postert T. Brain perfusion and ultrasonic imaging techniques. *Eur J Ultrasound* 2002; 16: 91-104
10. Feril LB, Kondo T, Zhao QL, Ogawa R, Tachibana K, Kudo N, Fujimoto S, Nakamura S. Enhancement of ultrasound-induced
apoptosis and cell lysis by echo-contrast agents. Ultrasound Med Biol 2003; 29: 331-337

11 Yamaya Y, Niizeki K, Kim J, Entin PL, Wagner H, Wagner PD. Effects of optison® on pulmonary gas exchange and hemodynamics. Ultrasound Med Biol 2002; 28: 1005-1013

12 Masugara H, Petcrs B, Lalitte S, Strachan MG, Ohmori K, De Marin AN. Quantitative assessment of myocardial perfusion during graded coronary stenosis by real-time myocardial contrast echo refilling curves. J Am Coll Cardiol 2001; 37: 262-269

13 Kitzman DW, Goldman ME, Gillam LD, Cohen JL, Aurigemma GP, Gottardi JS. Efficacy and safety of the novel ultrasound contrast agent perflutren (definity) in patients with suboptimal baseline left ventricular echocardiographic images. Am J Cardiol 2000; 86: 669-674

14 Driven HA, Rasmussen H, Johnson H, Videm S, Walday P, Grant D. Intestinal and hepatic lesions in mice, rats, and other laboratory animals after intravenous administration of gas-carrier contrast agents used in ultrasound imaging. Toxicol Appl Pharmacol 2003; 188: 165-175

15 Moran CM, Anderson T, Pye SD, Sboros V, McDicken WN. Quantification of microbubble destruction of three fluorocarbon-filled ultrasonic contrast agents. Ultrasound Med Biol 2000; 26: 629-639

16 Moran CM, Watson BJ, Fox KAA, McDicken WN. In vitro acoustic characterisation of four intravenous ultrasonic contrast agents at 30MHz. Ultrasound Med Biol 2002; 28: 785-791

17 Marelli C. Preliminary clinical experience in cardiology with Sonazoid. Am J Cardiol 2000; 86(Suppl): G10-13

18 Yokoyama N, Schwarz KQ, Chen X, Steinmetz SD, Becher H, Schimpky C, Schrief R. The effect of echo contrast agent on doppler velocity measurements. Ultrasound Med Biol 2003; 29: 765-770

19 Basude R, Duckworth JW, Wheatley MA. Influence of environmental conditions on a new surfactant-based contrast agent: ST68. Ultrasound Med Biol 2000; 26: 621-628

20 Porter TR, Xie F, Kricsfeld A, Kilzer K. Noninvasive identification of acute myocardial ischemia and reperfusion with contrast ultrasound using intravenous perfluoropropane-exposed sonicated dextrose albumin. J Am Coll Cardiol 1995; 26: 33-40

21 Bekeredjian R, Behrens S, Ruel J, Dinjus E, Unger E, Baum M, Kuecherer HF. Potential of gold-bound microtubes as a new ultrasound contrast agent. Ultrasound Med Biol 2002; 28: 691-695

22 Wei K, Crouse L, Weiss J, Villanueva F, Schiller NB, Naqviz TZ, Siegel R, Monaghan M, Goldman J, Aggarwal P, Feigenbaum H, Demaria A. Comparison of usefulness of dipyriramole stress myocardial contrast echocardiography to technetium-99m sestamibi single-photon emission computed tomography for detection of coronary artery disease (PB127 Multicenter Phase 2 Trial results). Am J Cardiol 2003; 91: 1293-1298

23 Bjerknes K, Braendtje JU, Skistad G, Agervikst E. Evaluation of different formulation studies on air-filled polymeric microparticles by multivariate analysis. Int J Pharma 2003; 257: 1-14

24 Basilico R, Blomley MJK, Harvey CJ, Filippone A, Heckemann RA, Eckersley RJ, Cosgrove DO. Which continuous US scanning mode is optimal for the detection of vascularity in liver lesions when enhanced with a second generation contrast agent? Euro J Radiol 2002; 41: 184-191

25 Bokor D. Diagnostic efficacy of Sonovue. Am J Cardiol 2000; 86(Suppl): 19G-24G

26 Totaro R, Baldassarre M, Sacco S, Marini C, Carolei A. Prolongation of TCD-enhanced doppler signal by continuous infusion of levovist. Ultrasound Med Biol 2002; 28: 1555-1559

27 Quaia E, Blomley MJK, Patel S, Harvey CJ, Padhani A, Price P, Cosgrove DO. Initial observations on the effect of irradiation on the liver-specific uptake of Levovist. Eur J Radiol 2002; 41: 192-199

28 Heckemann RA, Harvey CJ, Blomley MJK, Eckersley RJ, Butler-Barnes J, Jayaram V, Cosgrove D. Enhancement characteristics of the microbubble agent Levovist: reproducibility and interaction with aspirin. Eur J Radiol 2002; 41: 179-183

29 Liu P, Gao YH, Tan KB, Zuo S, Liu Z. Enhanced imaging of the rabbit liver using the self-made liposome contrast agent: an earlier experimental study. Zhongguo Chaosheng Yixue Zazhi 2003; 19: 4-6

30 Bouakaz A, Krenning BJ, Vletter WB, Cate FJ, Jong ND. Contrast superharmonic imaging: A feasibility study. Ultrasound Med Biol 2003; 29: 547-553

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