Ultrastructural Biologic Effects of Sonography with Pulse Inversion and Microbubble Contrast in Rabbit Liver

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ABSTRACT: *Purpose.* This prospective study was conducted to evaluate the biologic effects of microbubble destruction with pulse-inversion harmonic imaging on rabbit liver parenchyma.

Methods. The livers of 6 albino rabbits were examined sonographically by a single investigator. Three rabbits underwent contrast-enhanced sonography, with scanning starting 5 seconds after injection by using pulse-inversion harmonic imaging with a mechanical index of 1.2. Four time-triggered images were recorded at a rate of 1 frame every 2 seconds. For comparison, 3 control rabbits had pulse-inversion harmonic imaging with a mechanical index of 1.2 only, without contrast medium. Immediately after sonography, the animals were killed and uninterrupted, thin serial sections of the liver from both groups were analyzed by energy-filtered transmission electron microscopy.

Results. The hepatic parenchyma of rabbits exposed to contrast agents had ultrastructural damage: mitochondria with fragmented crests; interrupted rough endoplasmic reticulum; enlarged intercellular spaces; highly vacuolized cytoplasmic areas; dilated sinusoids, sometimes with an irregular and interrupted endothelial wall; fragmented hepatocyte microvilli in dilated spaces of Disse; fragmented or missing microvilli in bile canaliculi; vacuolated and lysosome-deprived hepatic cytoplasm around the bile canaliculi; markedly injured or fragmented endothelium in larger vessels; and damaged basal membrane. Control-group results indicated that exposure to ultrasound alone did not cause ultrastructural damage to hepatic cells.

Conclusions. Simultaneous exposure to contrast administration and pulse-inversion harmonic imaging with a high mechanical index causes ultrastructural damage in the rabbit liver. © 2005 Wiley Periodicals, Inc. *J Clin Ultrasound* **33**:106–111, 2005; Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jcu.20097

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To improve the quality of images, sonography takes advantage of contrast agents, consisting of microbubbles, and software that is able to induce the microbubbles to burst with a high mechanical index.[1] Since microbubbles are compressible, they alternately contract and expand in the acoustic field. At high acoustic pressure, the microbubbles are destroyed by means of inertial cavitation.[2] The process of cavitation can induce capillary damage to organs that contain air, such as the lungs,[3] or that are exposed to sonographic contrast agents.[2,4] Multiple experiments in laboratory animals have shown that the combination of microbubble contrast and ultrasound exposure can cause rupture of the vessels in which the microbubbles are located[4] and myocardial damage.[5,6]

We are not aware of any study investigating the ultrastructural biologic effects of sonographic contrast agents and pulse-inversion harmonic imaging (PIHI) on the liver parenchyma. On the basis of data obtained by other investigators in other organs,[4–6] we hypothesized that microbubble contrast agents

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may be associated with ultrastructural damage in the hepatic parenchyma. The purpose of our study, therefore, was to evaluate the biologic effects of microbubble destruction with PIHI in the rabbit liver parenchyma.

MATERIALS AND METHODS

Animals

For this prospective study, experiments were performed according to a protocol approved by our institutional animal care and use committee and in accordance with the guidelines issued by the National Research Council.[7]

Male rabbits (n = 6) were housed in a pathogen-free, temperature-controlled environment and allowed access to food and water ad libitum. Inbred 8-month-old male albino rabbits that weighed 1,500–1,700 g (mean, 1,600 g) were used in this study over a 3-day period. Although biopsy of the rabbit liver parenchyma was not performed before the experiments, we believe it is unlikely that these rabbits had liver steatosis because they were exposed to a controlled diet. Imaging was performed following induction of anesthesia by injection of Farmotol (0.25 mg of atropine and 60 mg of barbiturate; Farmitalia-Carlo Erba, Milan, Italy). The animal's skin was shaved after the animal was stabilized. Sonographic examinations were performed at the abdominal surface with the rabbit in the supine position.

Sonographic Examination Technique

A 5000 HDI ultrasound scanner (Philips-ATL, Bothell, WA) equipped with PIHI software and a C5-2 curved-array probe was used. The transducer was applied directly to the rabbits' skin without the use of any standoff pad. We used a scan depth of 6.7 cm and a single focus of 2.5 cm. To minimize interoperator variation, scanning was performed by a single radiologist (G.C.) with 15 years' experience using a single examination protocol.

Rabbits were divided into 2 groups of 3 rabbits each. Both groups underwent fundamental gray-scale sonography with optimized scanner settings for about 30 seconds, providing a baseline for identifying the area of the liver posterior to the gallbladder's longitudinal plane. We chose the portion of the liver posterior to the gallbladder to provide a landmark for pathologic examination that would allow good correlation between the area of insonation and the area analyzed on transmission electron microscopy.

Control group. Once the area of the liver posterior to the gallbladder was identified, in the control group continuous exposure to sonography (PIHI with a high mechanical index) for an additional 30 seconds (frame rate, 25 Hz) was performed in the same area of the liver that had undergone fundamental gray-scale sonography for 30 seconds. The control group was therefore exposed to fundamental gray-scale sonography for 30 seconds and to PIHI with a high mechanical index for an additional 30 seconds.

Study group. After fundamental gray-scale sonography for 30 seconds to identify the area posterior to the gallbladder, the rabbits in the study group received contrast agent injection and concomitant PIHI (Figure 1). Real-time scanning started 5 seconds after contrast injection. A suspension of galactose microparticles (99.9%) and palmitic acid (0.1%) in sterile water (Levovist, Schering, Berlin, Germany) was used as the sonographic contrast agent. When mixed with water, this agent produces microbubbles of air covered

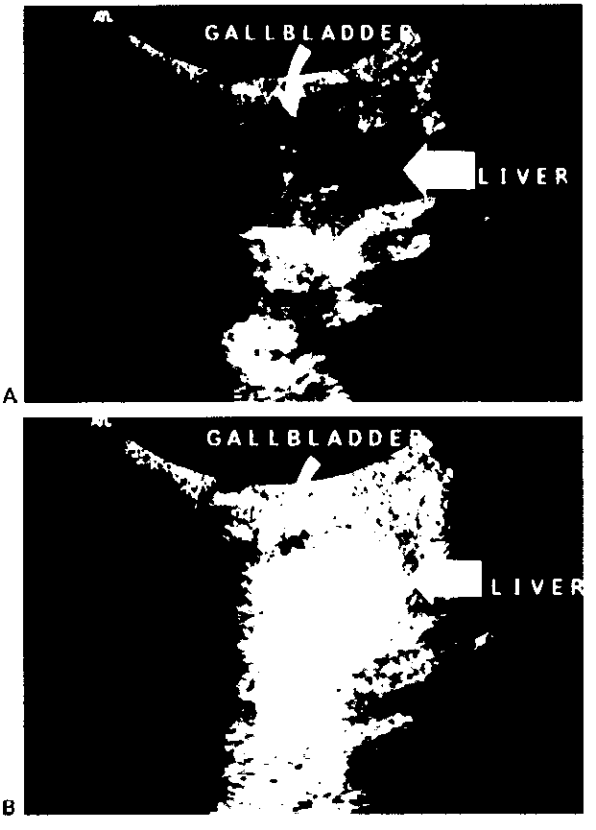

FIGURE 1. Sonograms of a rabbit liver before and after Levovist injection and pulse-inversion harmonic imaging. **(A)** Preinjection fundamental gray-scale sonogram shows the left lobe of the liver (arrow) and a normal anechoic gallbladder (curved arrow). **(B)** Sonogram obtained 5 seconds after contrast injection shows marked diffuse enhancement of the liver (arrow). The gallbladder is also indicated (curved arrow).

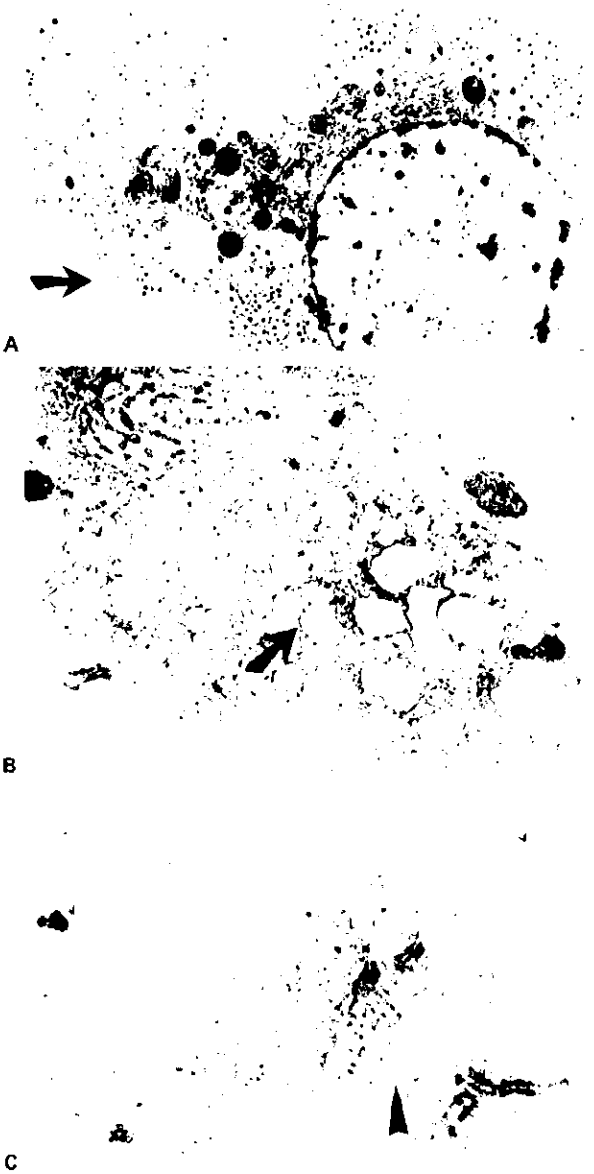

FIGURE 3. Comparison of ultrastructural appearances of hepatic sinusoids with and without Levovist injection. **(A)** Electron microscopy scan after ultrasound exposure in fundamental gray-scale sonography and pulse-inversion harmonic imaging, without Levovist injection shows normal sinusoid with endothelium and space of Disse (arrow) (original magnification, 5,000×). **(B)** Electron microscopy scan after injection of Levovist and pulse-inversion harmonic imaging shows vacuolized hepatic cytoplasm (arrow) (original magnification, 8,000×). **(C)** Electron microscopy scan after injection of Levovist and pulse-inversion harmonic imaging shows hepatic sinusoid with fragmented endothelium (arrowhead) (original magnification, 5,000×).

Disse was unalterated (Figure 3A); the intercellular spaces were undilated and capillary bile ducts appeared well conserved and delimited by the typical junction patterns, such as tight junctions and desmosomes (Figure 4A).

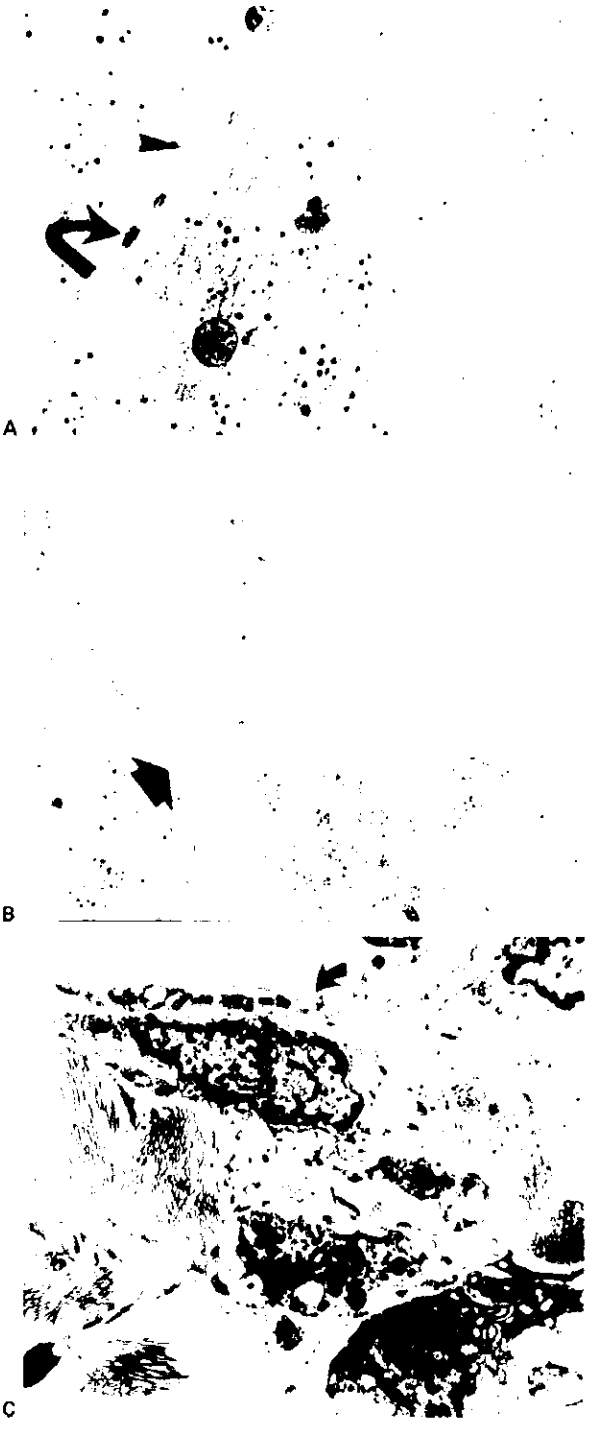

FIGURE 4. Comparison of ultrastructural appearances of bile canaliculi with and without Levovist injection. **(A)** Electron microscopy scan after ultrasound exposure in fundamental gray-scale sonography and pulse-inversion harmonic imaging without Levovist injection shows normal bile canaliculi (arrowhead) and junctional complex (curved arrow) (original magnification, 8,000×). **(B)** Electron microscopy scan after injection of Levovist and pulse-inversion harmonic imaging shows dilated bile canaliculi (arrow); the normal junctional complexes are no longer seen (original magnification, 6,000×). **(C)** Electron microscopy scan after injection of Levovist and pulse-inversion harmonic imaging shows fragmented endothelium and interrupted basal membrane (curved arrow) (original magnification, 6,000×).

Study Group

In the group of rabbits exposed to fundamental gray-scale sonography and ultrasound contrast agent injection with PIHI, the hepatocytes presented mitochondria with fragmented crests and interrupted rough endoplasmic reticulum, which was distributed irregularly among the mitochondria. The intercellular spaces were enlarged (Figure 2B). Some cytoplasmic areas appeared highly vacuolized (Figure 3B); sinusoids appeared dilated, and their endothelial wall was sometimes irregular and interrupted (Figure 3C). Hepatocyte microvilli were often sprained or fragmented in a dilated space of Disse. Bile canaliculi showed fragmented and missing microvilli. The hepatic cytoplasm around the bile canaliculi was vacuolated and lysosome deprived. Bile canaliculi junction patterns were not well structured (Figure 4B). In larger vessels, the endothelium was markedly injured or fragmented; the basal membrane was damaged (Figure 4C).

DISCUSSION

The findings in this study support our hypothesis that the interaction between ultrasound and microbubbles may cause ultrastructural damage of the liver, consisting of intracellular and capillary damage, enlargement of the intercellular spaces, and fragmentation of the bile canaliculi.

A potential limitation of our study could have been a lack of correspondence between the area of insonation and that subsequently analyzed at pathology. We tried to minimize the potential for bias by using the gallbladder as a landmark. Moreover, since it is known that hepatic cells can be damaged by some anesthetics, we were particularly careful to choose a drug with minimal hepatotoxic effects.

Our study findings suggest that sonography contrast agents induce ultrastructural alterations of the cell organelles and blood vessels in the rabbit liver. We speculate that these alterations are induced by microbubble rupture with immediate release of mechanical and thermal energy.[2] No alterations were found in the control group of rabbits that were exposed to sonography only, without injection of microbubble contrast agent. Ay et al[6] showed that isolated rabbit hearts exposed to ultrasound and contrast agents had capillary rupture. Skyba et al[4] found that the amounts of capillary damage and microbubble destruction were proportional to the mechanical index. Our results are in agreement with those reported by these authors and extend these observations to the rabbit liver. Similar to the rupture of microvessels within muscles,[4] we observed the rupture of hepatic sinusoids and larger vessels in rabbit livers. The disruption of microbubbles in sinusoids causes such alterations as the development of numerous vacuoles, rupture of the endothelium, and damage of the basal membrane. We obtained our results in an in vivo model, while a previous study obtained similar results in an in vitro model.[6] It is noteworthy that the studies by Ay et al,[6] Skyba et al,[4] and Chen et al[5] included morphologic analysis with a light microscope, while we used a transmission electronic microscope, allowing us to investigate damage at the ultrastructural level.

Our study has several limitations. First, although the use of animal models that mimic the sequence of events in humans is essential for evaluating the biologic effects of sonographic contrast media and PIHI, it is difficult to make direct comparisons between results from animal studies and observations in human clinical studies. It is possible that the rabbit liver responds differently than human liver to the association of Levovist and PIHI. Second, in this study we could not evaluate the long-term consequences of this technique on the hepatic cells or any changes in liver function test results because the animals were killed immediately after sonography. Third, the scanning time, which began 5 seconds after contrast injection, was empirical; different results may have been obtained with later or earlier scanning. Fourth, the number of animals was small. Although not acceptable for a formal statistical analysis, the data from our small sample of 3 study animals is noteworthy. Since the experimental results of our study have important implications, further studies with larger study groups should be performed to confirm the effects and to assess the long-term consequences of simultaneous exposure to Levovist and PIHI on the liver parenchyma. Finally, we did not evaluate whether contrast medium alone, without insonation, might have damaged the liver parenchyma. However, to our knowledge the literature contains no reports of a potential bioeffect of contrast medium alone.

Our study raises many potential research questions. It would be interesting to investigate whether a lower mechanical index evaluation of different rabbits or another portion of the rabbit liver would find any effects on the liver parenchyma. Also, it would be interesting to let the rabbits live for several weeks after sonography to see if any residual damage would be found. Moreover, the design of our study prevented us from evaluating the percentage of tissue damage in the insonated liver, as we classified damage as present or absent only; thus, effects related to the degree of damage are unknown. Prospective studies should be performed to answer these questions.

In conclusion, despite the relatively few animals used in this study, our results suggest that simultaneous exposure to Levovist and PIHI causes ultrastructural damage in the rabbit liver. Although similar effects may be expected in humans, additional and more extensive experimental studies should be performed to confirm our preliminary findings.

REFERENCES

1. Correas JM, Bridal L, Lesavre A, et al. Ultrasound contrast agents: properties, principles of action, tolerance and artefacts. Eur Radiol 2001;11:1316.
2. Church CC, Carstensen EL. "Stable" inertial cavitation. Ultrasound Med Biol 2001;27:1435.
3. Dalecki D, Child SZ, Reaman CH, et al. Age-dependence of ultrasonically-induced lung hemorrhage in mice. Ultrasound Med Biol 1997;23:767.
4. Skyba DM, Price RJ, Linka AZ, et al. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. Circulation 1998;4:290.
5. Chen S, Kroll MH, Shohet RV, et al. Bioeffects of myocardial contrast microbubble destruction by echocardiography. Echocardiography 2002;19:495.
6. Ay T, Havaux X, Van Camp G, et al. Destruction of contrast microbubbles by ultrasound: effects on myocardial function, coronary perfusion pressure, and microvascular integrity. Circulation 2001 24;104:461.
7. Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council. Guide for the care and use of laboratory animals. Washington, DC: National Academy Press; 1996.