

# Contrast-Enhanced Diagnostic Ultrasound Causes Renal Tissue Damage in a Porcine Model

Douglas L. Miller, PhD, Chunyan Dou, MD,  
Roger C. Wiggins, MB, BChir, MA

**Objective.** Glomerular capillary hemorrhage (GCH) has been reported and confirmed as a consequence of contrast-enhanced diagnostic ultrasound (CEDUS) imaging of rat kidney. This study assessed renal tissue injury in the larger porcine model. **Methods.** The right kidneys of anesthetized pigs were imaged in 8 groups of 4 pigs. A Vingmed System Five ultrasound machine (General Electric Co, Cincinnati, OH) was used at 1.5 MHz in the B-mode to intermittently scan the kidney at 4-second intervals. An Acuson Sequoia 512 machine (Siemens Medical Solutions, Mountain View, CA) was used in the 1.5-MHz Cadence contrast pulse sequencing mode with intermittent agent clearance bursts at 4-second intervals. Kidneys were scanned transabdominally or after laparotomy through a saline stand-off. The second machine's probe was placed in contact with the kidney for 1 group. A perflutren lipid microsphere contrast agent (Definity; Lantheus Medical Imaging, Inc, North Billerica, MA) was infused at 4  $\mu\text{L}/\text{kg}/\text{min}$  (diluted 33:1 in saline) for 4 minutes during scanning. **Results.** Blood-filled urinary tubules were evident on the kidney surface for all groups except the group with the probe in contact with the kidney. Glomerular capillary hemorrhage was found by histologic processing in  $31.7\% \pm 9.8\%$  (mean  $\pm$  SD) of glomeruli in the center of the scan plane for 1.7-MPa transabdominal scanning and  $1.5\% \pm 2.9\%$  of glomeruli in sham samples ( $P < .05$ ). In addition, hematuria was detected after scanning, and tubular obstruction occurred in some nephrons. **Conclusions.** Renal tissue damage was induced by CEDUS in the porcine model. This result, together with previous studies in rats, support a hypothesis that GCH would occur in humans from similar CEDUS exposure. **Key words:** Bowman's space clot; diagnostic ultrasound adverse effects; glomerular hemorrhage; renal intratubular obstruction; ultrasound contrast agent.

## Abbreviations

CEDUS, contrast-enhanced diagnostic ultrasound; GCH, glomerular capillary hemorrhage; IV, intravenous; MI, mechanical index; Mle, mechanical index equivalent; PRPA, peak rarefactional pressure amplitude

Received March 16, 2010, from the Departments of Radiology (D.L.M., C.D.) and Internal Medicine, Division of Nephrology (R.C.W.), University of Michigan Health System, Ann Arbor, Michigan USA. Revision requested April 7, 2010. Revised manuscript accepted for publication June 7, 2010.

We thank Jonathan M. Rubin, MD, PhD (University of Michigan), for the loan of the Sequoia 512 ultrasound system during out-of-service periods. This work was supported by Public Health Service grant EB00338, awarded by the National Institutes of Health, US Department of Health and Human Services.

Address correspondence to Douglas L. Miller, PhD, University of Michigan Medical Center, Room 3315, Kresge III, 200 Zina Pitcher Pl, Ann Arbor, MI 48109-0553 USA.

E-mail: douglm@umich.edu

Contrast-enhanced diagnostic ultrasound (CEDUS) has shown great promise for augmenting the information available from ultrasound examinations. Ultrasound contrast agents are injectable suspensions of gas bodies (stabilized microbubbles). The gas bodies are scientifically engineered to persist in the circulation and provide a strong echo return for image enhancement.<sup>1</sup> Under some conditions, the gas bodies can also serve as near-optimal nuclei for ultrasonic cavitation, which can lead to localized tissue damage.<sup>2</sup> Contrast-enhanced diagnostic ultrasound methods that have been associated with a risk of bioeffects involve intermittent imaging at relatively high diagnostic pulse amplitudes. These modes use gas body destabilization and destruction for imaging of perfusion

either by “flash” images at different intervals or by agent-destructive exposure followed by non-destructive imaging of the refill of the imaged tissue with blood containing gas bodies. The agent-destructive pulses can induce ultrasonic cavitation behavior for some gas bodies within capillaries, leading to capillary rupture and tissue injury, which have been observed in heart, kidney, and other tissues.<sup>2</sup>

Capillary rupture in kidney glomeruli is particularly important because of the unique structure of the kidney.<sup>3,4</sup> Because of the high kidney blood flow, the concentration of gas bodies is higher in the kidney, especially in the glomerular tuft, than in most other tissues. Glomerular capillary hemorrhage (GCH) flows into Bowman’s space and urinary tubules, which amplifies the impact of the capillary rupture by affecting the entire nephron functional unit. The relatively high hydrostatic pressure of glomerular capillaries can result in sufficient hemorrhage to precipitate fibrin formation in Bowman’s space, intratubular obstruction, and acute tubular necrosis.<sup>5,6</sup> Because of the similarity of kidney structure in different mammalian species, the observation of GCH in rats also implies that similar effects would occur in other species, including humans, under similar conditions of CEDUS.<sup>5</sup>

Our previous studies using rats involved B-mode intermittent CEDUS with a phased array probe.<sup>4</sup> To simulate human scanning, the rats were scanned in a water bath with the ultrasound focal zone placed in the kidney. Glomerular capillary hemorrhage was demonstrated for a range of examination scan conditions. Recently, Jiménez et al<sup>7</sup> reported that CEDUS in pig kidney did not cause renal tissue damage. For that study, the kidney was scanned with a curved linear array probe using a contrast-specific mode and intermittent scan bursts for contrast clearance. The kidneys were accessed by laparotomy, and the probe was placed in contact with the kidney surface. The rationale for contact scanning of the kidney was not clear, particularly because the method does not allow clear imaging of the proximal cortex for normal settings of the focal zone depth. No visual or histologic evidence of GCH in the proximal cortex or other renal tissue damage was found in this porcine model of CEDUS.<sup>7</sup> These contradictory results for rats and pigs were

puzzling and worthy of further investigation. This study was undertaken to assess possible renal tissue damage in pigs using methods that replicated, as much as possible, the methods that would be used in humans. In addition, methods similar to those of Jiménez et al<sup>7</sup> were tested to understand the origin of the contradictory results.

## Materials and Methods

### *Animal Preparation*

This investigation was conducted with the approval of the University Committee on the Use and Care of Animals of the University of Michigan. A total of 48 female pigs (crossbred Yorkshire and Landrace breeds), which weighed  $27.4 \pm 3.0$  kg, were obtained from Michigan State Swine Research (East Lansing, MI). Pigs were fasted for 12 hours before beginning testing. The induction anesthetic was 6 mg/kg tiletamine/zolazepam plus 2.2 mg/kg xylazine injected intramuscularly. The pigs were maintained at the desired level of anesthesia by 1% to 3% isoflurane in oxygen, which was delivered via a breathing bag and snout cone. Atropine (0.05 mg/kg intramuscularly) was given to control respiratory secretions. For intravenous (IV) injections and sterile saline infusion, 22-gauge cannulas were inserted into a vein on both ears. Infusion of 0.9% sodium chloride from 1-L bags via a primary IV line was continuous throughout all procedures. The pigs were placed on their left side, and vital signs were monitored with a veterinary monitor (Vet/Ox Plus 4800; Sims BCI, Inc, Waukesha, WI). Body temperature maintenance was aided by the use of warming pads and insulating drapes. Average values and SDs at the time of scanning were as follows: oxygen saturation,  $96 \pm 2$ ; heart rate,  $113 \pm 23$  beats per minute; respiration rate,  $37.5 \pm 9.6$  breaths per minute; and temperature,  $36.8^\circ\text{C} \pm 0.9^\circ\text{C}$ . For transabdominal scanning, the skin over the right kidney was cleaned and depilated. For direct access to the right kidney, a laparotomy was performed to expose the kidney surface without breaching the peritoneum. This allowed scanning of the kidney with the normal tissues in place around it, other than the abdominal wall. Retractors were used to produce an opening of about  $8 \times 10$  cm,

but, typically, several centimeters of the kidney remained hidden under the lowest ribs. This method differed from that of Jiménez et al,<sup>7</sup> in which the intestines were moved, and the abdominal cavity was filled with saline for complete access to the kidneys.

The ultrasound contrast agent for this study was Definity (perflutren lipid microsphere injectable suspension; Lantheus Medical Imaging, Inc, North Billerica, MA). A fresh vial was prepared each day according to the manufacturer's instructions. For infusion, 0.6 mL of prepared Definity was diluted in 20 mL of sterile saline in a syringe, which was connected to an ear vein cannula with an extension tube. The diluted agent was infused at 0.14 mL/kg/min with a syringe pump (Aladdin 1000; World Precision Instruments, Sarasota, FL), which delivered 16  $\mu$ L/kg Definity in 4 minutes at a 4- $\mu$ L/kg/min rate (approximating the infusion method and maximum rate specified in the package insert). The use of Definity allows for comparison of results to previous study results in rats. Jiménez et al<sup>7</sup> used SonoVue (sulfur hexafluoride microsphere suspension; Bracco SpA, Milan, Italy), which at this time has not been approved for clinical use in the United States. Unfortunately, Bracco representatives declined to provide SonoVue for this investigation, which would have allowed a more direct comparison to the study by Jiménez et al.<sup>7</sup>

During initial tests, some pigs had anaphylactoid-like reactions to the contrast agent, which included erythema, bradycardia, and reduced respiration, after 1 to 2 minutes of agent infusion. Such reactions are known to occur from ultrasound contrast agents, particularly in pigs, including Definity,<sup>8</sup> Albunex (human albumin; Nycomed Imaging AS, Oslo, Norway),<sup>9</sup> and SonoVue.<sup>10</sup> Both Definity and SonoVue have associated warnings concerning possible human reactions. Pigs were given IV injections of 1 mg/kg diphenhydramine and 2 mg of dexamethasone about 1 hour before CEDUS as a preventive measure. This type of preventive strategy has also been used for other radiologic contrast media reactions.<sup>11,12</sup>

Urinalysis was conducted to determine whether hematuria, which was detected in rats,<sup>5</sup> also occurred in swine. For rats, the animals were

placed in urine collection cages, and the urine sample was collected after 4 hours. This method could not be used for the swine. The cannulation of the urethra was very difficult in these animals and could not be used for urine samples. In 1 group, urine samples were collected before and 2 hours after exposure by transabdominal puncture of the bladder using a syringe and a 21-gauge needle. This method was poor because it involved dilution of any postexposure blood in a variably filled bladder. For another group of pigs with transabdominal exposure, the right ureter was accessed by laparotomy, and a 3.6-mm-diameter cannula was inserted for urine sampling at 10-minute intervals before, during, and 3 times after exposure. This method was also difficult, and some blood was present in initial samples because of the cannulation procedure. Preexposure sampling of urine was done to ensure a negative hematuria reading before exposure. The hematuria was read from Hemastix (Bayer Corp, Elkhart, IN), and a hematuria score was calculated as described previously.<sup>5</sup>

### **Diagnostic Ultrasound**

Two diagnostic ultrasound machines were used for exposure-scanning. The first machine was a Vingmed System Five (General Electric Co, Cincinnati, OH) with an FPA2.5 phased array probe. This probe was protected with a probe cover (Cone Instruments, Solon, OH), clamped in an adjustable support stand, and aimed downward at the kidney. A bag, which was made from a 14-cm-wide polyurethane ultrasound probe cover, was secured over the end of the probe and filled with saline to allow variation in the distance between the probe and the kidney cortex. The acoustic coupling of the bag to the kidney was by saline, and coupling to the skin was by ultrasound coupling gel and saline. The distance from the probe to the kidney was adjusted to place the proximal cortex in the focal zone 3.5 to 4.5 cm from the transducer face where the -6-dB scan plane thickness (beam width) was 4.3 mm. The machine was set to 1.5 MHz, 5-cm focus, 10-cm depth, and a 4-second interval between dual scans (50 milliseconds apart). Dual scans were used to show contrast destruction by the loss of enhanced contrast in the second image. This gave 60 scans during 4

minutes of agent infusion, which was intended to parallel the use of 60 scans during 1 minute of agent infusion in the previous studies with rats.<sup>4</sup> The peak rarefactional pressure amplitude (PRPA) of the 1.5-microsecond pulses (about 2 cycles) was measured in a water bath with a calibrated hydrophone (model 805; Sonora Medical Systems, Inc, Longmont, CO). At the maximum (0-dB) setting, the PRPA was 2.3 MPa. Each scan had a pulse amplitude sequence previously found to be 4.6 milliseconds in duration.<sup>13</sup> For transabdominal scanning, the PRPA at the kidney was estimated by inserting samples of the abdominal wall between the probe and hydrophone. Four 10 × 15-cm samples of the abdominal wall over the kidney were taken from a group with transabdominal exposure and measured at 4 different positions to obtain a nominal (average of the 4 measurements) reading for each sample. The mean PRPA was  $1.66 \pm 0.14$  MPa for the mean thickness of  $1.9 \pm 0.2$  cm. Comparing this result to the water PRPA value gives an attenuation of  $-2.8$  dB (a factor of 0.73) or about 1.0 dB/cm/MHz for the skin plus muscle tissues. The on-screen mechanical index (MI) was 1.7 for the 0-dB setting. The measured mechanical index equivalent (MIe; given by the PRPA divided by the square root of the frequency) was  $MIe = 1.9$  for the water path and  $MIe = 1.4$  for the transabdominal path.

The second diagnostic ultrasound machine was an Acuson Sequoia 512 (Siemens Medical Solutions, Mountain View, CA), which was the same model machine used by Jiménez et al.<sup>7</sup> A 4C1-S probe was used in the 1.5-MHz Cadence contrast pulse sequencing mode, which is a contrast-specific mode, with a 10-cm image depth and foci at 4 and 6 cm. This probe also was protected with a probe cover and was clamped for kidney scanning through a saline-filled bag as described above. The repeated burst mode was used for clearance of the contrast agent from the image with 5 frame bursts (the default setting), which totaled 110 milliseconds in duration, each 4 seconds. Low-power imaging with a 1-second interval and a PRPA of 0.1 MPa occurred between the clearance bursts. This was below the exposure levels required for gas body destabilization or GCH, so the low-power imaging can be neglected with regard to the GCH bioeffect. The

exposure timing was therefore essentially the same as for the Vingmed System Five exposure (ie, intermittent high-power agent-destructive bursts at 4-second intervals). The maximum PRPA was measured in the water bath to be 1.9 MPa for the 3.06-microsecond pulses (about 5 cycles) in the clearance burst. The actual duration of 5 cycles was 2.77 microseconds, which represents an ultrasound frequency of 1.8 MHz. For this setting, the burst MI was listed at 1.9 on screen, but the measured MIe was 1.4 even for the water path. The reason for this discrepancy between the output expected according to the on-screen MI and the measured values was uncertain. The MI value of 1.9 is the US Food and Drug Administration guideline upper limit for diagnostic ultrasound. However, the measured values were consistent with the information given in the transducer specification manual for this probe, which listed the maximum MI as 1.2. For 1 group, the saline-filled bag was omitted, and the probe was placed in contact with the kidney, which was the method used by Jiménez et al.<sup>7</sup> This method necessitated some pressure on the kidney by the curved array to increase the width of the contact region in the image, but the ends of the probe typically were not in contact. The largest rarefactional pressure amplitude was 0.96 MPa at 0.5 cm and 1.05 MPa at 1.5 cm, which included the proximal cortex (ie, the focal zone and PRPA were deeper into the kidney in the medulla or distal portions of the cortex).

In addition, the wash-in and wash-out of contrast enhancement were observed in the left kidney for 1 group of pigs using flash echo imaging with a PowerVision 8000 ultrasound machine (model SSA-390A; Toshiba Corporation, Tochigi-Ken, Japan) with a curved linear array (PVN-375AT). This procedure was used previously for assessing the loss of circulating contrast agent gas bodies in rats due to natural loss mechanisms and to contrast agent destruction by exposure of the right kidney.<sup>14</sup> For our study, this method was used to assess the gas body concentration in the larger animal for comparison to the results in rats. The image depth was 8 cm with a focus of 6 cm for the harmonic imaging mode (2.3 MHz transmit and 4.6 MHz receive). A region of interest was selected in the proximal renal cortex, and time intensity-curves for 4 minutes dur-

ing infusion and 4 minutes after infusion were obtained twice, once without and once with exposure-scanning of the right kidney. The curve data were collected at 10-second intervals. The time-intensity curves were evaluated by nonlinear curve fitting to obtain the plateau level and the decay (wash-out) time constant, as described previously.<sup>14</sup>

### **Measured End Points**

At the conclusion of testing, the animals were euthanized while under anesthesia, and the right kidney was removed. The kidney surface was photographed after removal of the renal capsule. Exposed kidney samples were taken for histologic processing from the scan plane identified by blood-filled urinary tubules visible on the surface, and an adjacent sham sample was taken outside the scanned region of each kidney. All scans were approximately parallel to the sagittal plane to provide a longitudinal view of the kidney, which was the orientation used by Jiménez et al.<sup>7</sup> For open-abdomen exposure, the observable band of blood-filled tubules was 6 to 7 cm long, and 4 exposed samples were collected. For transabdominal exposure, the ribs restricted the view of the kidney to a greater extent, so that the observable band was 4.5 to 6 cm long, and 3 or 4 exposed samples were collected. For scanning with the probe in contact with the kidney, the scan plane was marked after euthanasia by making small cuts on either side of the probe. No blood-filled tubules were seen on the proximal side of the kidney for contact scanning, but some were seen on the distal side: 2 samples of cortex were collected from the proximal side and 2 from the distal side for this group. Kidney samples were immersion fixed in neutral buffered formalin and trimmed for placement in histologic cassettes. Histologic processing was performed at the Research Histology and Immunoperoxidase Laboratory of the University of Michigan Comprehensive Cancer Center Tissue Core. Glomerular capillary hemorrhage was evaluated by histologic processing with hematoxylin and eosin staining as described previously.<sup>4</sup> Glomeruli with and without evidence of hemorrhage were counted, and the counts were used to calculate the percentage of GCH. Results for the 2, 3, or 4 exposed samples were summed to

represent the exposed region.

In rats, intratubular obstruction was shown to occur from CEDUS by measurements of the width of Bowman's space and detection of fibrin in the urinary space.<sup>6</sup> Possible intratubular obstruction in the swine kidney was assessed, using the histologic methods described previously.<sup>6</sup> In addition, antibody staining for fibrin was used to identify clots in glomeruli for the 2 groups with delayed euthanasia (30 minutes and 4 hours) for urine collection. Fibrin staining was performed on serial sections adjacent to the hematoxylin and eosin-stained slides used for GCH scoring. A rabbit antihuman antibody to fibrinogen (Dako North America, Carpinteria, CA) was used to tag the clots, followed by peroxidase-labeled antirabbit antibody and diaminobenzidine staining. Negative controls were run on separate slides using a normal rabbit immunoglobulin fraction. The slides stained for fibrin were scored for the percentage of glomeruli with clearly identifiable clots, which stained dark brown.

### **Experimental Plan and Statistics**

A total of 48 pigs were obtained for this study. Seven of these were used in preliminary testing to develop the best methods for the research. The preventive treatment noted above appeared to help prevent mild reactions, such as erythema, but 4 pigs had cessation of respiration or the heartbeat during agent infusion and were lost from the study. These severe reactions evince the failure of the preventive pretreatment (see "Materials and Methods") to eliminate anaphylactoid reactions. In addition, results from 5 pigs were excluded because of technical problems with performing the experiment (eg, movement of the animal). The final study results included 8 separate groups of 4 pigs each, which are listed in Table 1. Numerical results are presented as the mean  $\pm$  SD or plotted as the mean with SE bars. For statistical analysis, Mann-Whitney rank sum tests were used to compare means of the measured parameters, with statistical significance assumed at  $P = .05$ . For multiple comparisons, 1-way analysis of variance was performed with pair-wise comparisons made using the Holm-Sidak method.

**Table 1.** Test Groups for Which a Standoff Was Normally Used to Place the Focal Zone in the Proximal Renal Cortex

Group	Ultrasound System	Probe Placement	PRPA, MPa	Euthanasia Delay	Test End Points	Exp GCH, %	Shm GCH, %	P
1	Vingmed	Open, so	1.3	<5 min	GCH	0.3 ± 0.4	0 ± 0	NS
2	Vingmed	Open, so	1.7	<5 min	GCH	26.5 ± 10.5	0 ± 0	<.005
3	Vingmed	Open, so	2.3	<5 min	GCH, B, T	59.2 ± 2.2	1.1 ± 2.2	<.001
4	Vingmed	Open, so	2.3	2 h	GCH, F, H	26.6 ± 10.2	0 ± 0	<.05
5	Vingmed	Closed, so	1.7	<5 min	GCH	29.6 ± 11.5	1.5 ± 2.9	<.05
6	Sequoia	Open, so	1.9	<5 min	GCH	42.7 ± 2.8	0 ± 0	<.05
7	Sequoia	Cont, prox	0.96–1.05	<5 min	GCH	0.06 ± 0.11	0 ± 0	NS
		Cont, dist	ND			6.6 ± 4.9		<.05
8	Sequoia	Closed, so	1.3	30 min	GCH, F, H	7.9 ± 5.6	0 ± 0	<.05

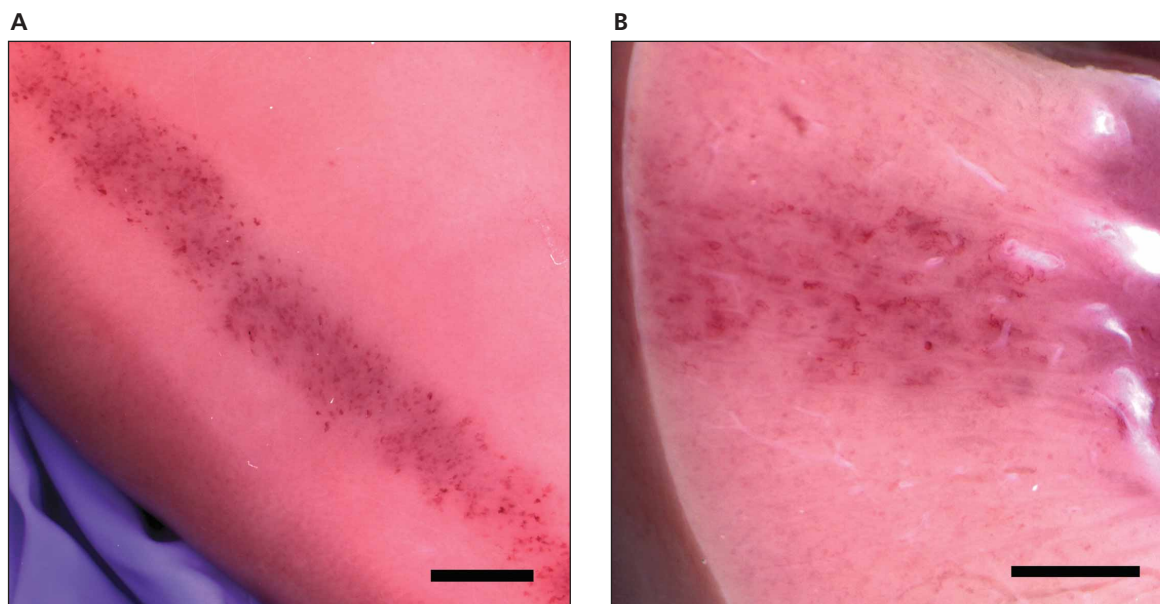
For Sequoia exposure with probe contact, the rarefactional pressure amplitude in the proximal cortex is given (rather than the PRPA), and for the closed-abdomen exposure, the PRPA was derated by the 1.0-dB/cm/MHz attenuation measured for the intervening abdominal wall. Closed indicates closed abdomen; Cont, contact; dist, distal; Exp, exposed; Open, open abdomen; ND, not determined; NS, not significant; prox, proximal; Shm, sham; and so, standoff. The end points are GCH, Bowman’s space measurement (B), time-intensity analysis (T), immunohistologic fibrin staining (F), and testing for hematuria in urine samples (H).

**Results**

A band of blood-filled tubules due to GCH was typically seen on the surface of the kidneys. Figure 1 shows the hemorrhage marks in the scan plane and a cross section of the cortex for one of the kidneys of group 5. The percentage of GCH (glomeruli containing red cells within Bowman’s capsule) at the approximate center of the scan plane for each group is listed in Table 1, together with the GCH in the adjacent sham

sample and P values comparing each set of sham and exposed data. Some sham samples had evident GCH in histologic samples (presumably these samples were not completely outside the scanned volumes), but the sham GCH results were not significantly different from 0. The results for groups 1, 2, and 3 with open-abdomen exposure of the kidney through a saline standoff form a small exposure-response test, as shown in Figure 2. Closed-abdomen group 5 was added to this plot because the attenuation through the

**Figure 1.** Band of marks seen on the kidney surface, resulting from hemorrhage into urinary tubules, on a group 5 kidney with closed-abdomen imaging-exposure (A) and cross section cut through the band to show the penetration of tissue injury through the cortex (B). Scale bars indicate 5 mm (A) and 2 mm (B).

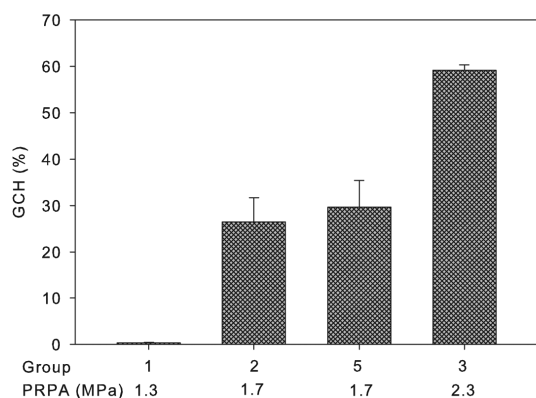


abdominal wall approximated the reduction ( $-3$  dB) from maximum output for group 2, and the GCH results are essentially the same for these 2 groups. The lowest PRPA exposure (group 1) had nonsignificant GCH in the histologic specimens but did show blood-filled tubule markings on the surface of each exposed kidney, with 8, 5, 8, and 8 marks in the 4 kidneys, respectively. The blood-filled tubule markings are a more sensitive test for low effects, as noted previously.<sup>15</sup> The mean of  $7.3 \pm 1.5$  marks was significantly greater ( $P < .05$ ) than the 0 effect outside the scan plane.

The width of Bowman's space was determined for group 3 for the sham and exposed samples. For the exposed samples, glomeruli were categorized as having the Bowman's space empty ( $40.9\% \pm 2.4\%$ ), partially full ( $44.4\% \pm 3.7\%$ ), and essentially full ( $14.7\% \pm 3.4\%$ ) of erythrocytes. The results are shown in Figure 3. Pair-wise comparisons indicated that the mean widths for partial and full categories of the exposed samples were significantly larger than for sham samples ( $P < .01$ ), and the mean widths for the sham and empty scores were not significantly different. The substantial enlargement of Bowman's space was indicative of intratubular obstruction.

Time-intensity curves were generated from the left kidney of each pig in group 3, 1 without exposure of the right kidney and 1 with exposure. The plateau values of  $22.1 \pm 18.7$  relative units without and  $23.2 \pm 18.3$  relative units with exposure of the right kidney were not significantly different.

**Figure 2.** Mean percentage with SE bars of GCH induced by the Vingmed System Five machine in the center of the scan plane for groups 1, 2, 3, and 5 listed in Table 1 with the specified PRPA for the scans.

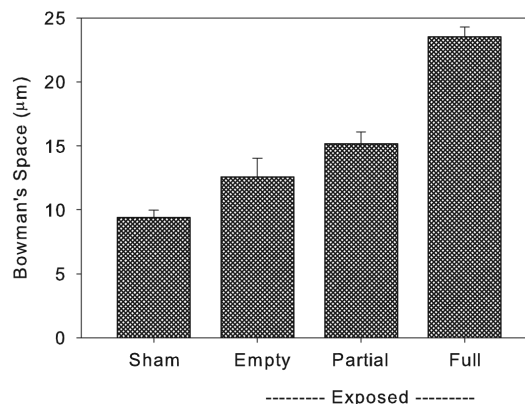


This indicated that the destruction of the contrast agent in the right kidney during scanning (ie, exposure) did not substantially reduce the circulating dose of the contrast agent. The intensity decay constants after the end of infusion were  $26.0 \pm 6.0$  and  $28.4 \pm 11.3$  seconds (not significantly different) with and without exposure of the right kidney, respectively.

Group 4 was scanned in the same way as group 3, but euthanasia was delayed for 2 hours to allow for urine collection and fibrin formation in Bowman's space. This group had a GCH score of  $26.6\% \pm 10.2\%$ , which indicated that many glomeruli had not cleared the hemorrhage by 2 hours. Slides stained for fibrin showed that  $34.1\% \pm 21.7\%$  of glomeruli had evidence of fibrin formation, which was indicative of activation of the coagulation system in Bowman's space. A few glomeruli ( $5.3\% \pm 6.8\%$ ) had fibrin nearly filling Bowman's space and often extending into the tubule, as shown in Figure 4. Urine collections from the full bladder for this group showed test strip results that were negative for hematuria before scanning and positive for hematuria after scanning ( $P < .05$ ).

Three groups of pigs were scanned with the Sequoia 512 machine, as listed in Table 1. Group 6 with open-abdomen exposure and the saline standoff gave  $42.7\% \pm 2.8\%$  GCH, which was commensurate with the results for the Vingmed

**Figure 3.** Measurements of the width of Bowman's urinary space (between the glomerular tuft and capsule) for samples from group 3 pigs. Exposed Bowman's spaces were measured for erythrocyte content categorized as empty, partially full, and essentially full of hemorrhage. The enlargement of Bowman's space is indicative of intratubular obstruction by the hemorrhage into the urinary space and tubules.

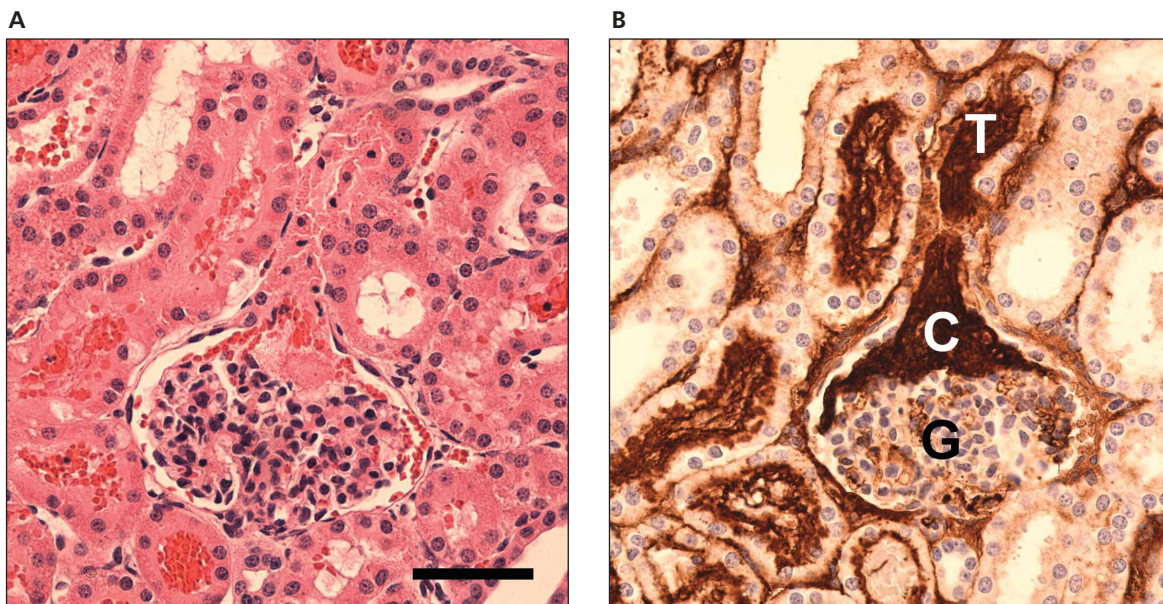


machine. The hemorrhage marks for one of the group 6 kidneys is shown in Figure 5. Group 7 with open-abdomen exposure and scanning with the probe in contact with the kidney (ie, without a standoff) had 1 GCH detected in 4 pigs, which was not significant. The different ultrasound images obtained by the contact and closed-abdomen scanning are shown in Figure 6. For the contact scanning, the machine had difficulty forming an image near the probe (the focal points remained at 4 and 6 cm, as for the other groups), and there was no clear indication of enhanced contrast within the proximal cortex. However, there were indications of enhanced contrast in the distal cortex, and samples taken from the distal side of the kidneys had  $6.6\% \pm 4.9\%$  GCH ( $P < .05$ ). Group 8 with transabdominal scanning had  $7.9\% \pm 5.6\%$  GCH ( $P < .05$  relative to 0 in sham samples) in the proximal cortex. Fibrin staining was done on adjacent serial sections, and  $4.5\% \pm 2.7\%$  of glomeruli had evidence of clotting in Bowman's space ( $P < .05$ ). Urine collections for this group were positive for blood for the samples collected 10 and 20 minutes after exposure ( $P < .05$  relative to negative samples before and during exposure) but were inconsistent (not statistically significant) for the 30-minute collection.

### Discussion

Contrast-enhanced diagnostic ultrasound was investigated in swine with regard to production of renal tissue damage. Glomerular capillary hemorrhage (Table 1 and Figures 1, 2, 4, and 5) was produced with open- or closed-abdomen exposure and was similar to the effect previously found in rat kidneys. In rats, CEDUS involved  $10\text{-}\mu\text{L}/\text{kg}/\text{min}$  infusion for 1 minute and 1-second intermittent imaging-exposure using the Vingmed System Five. Glomerular capillary hemorrhage was found to be 0% at 0.8 MPa,  $5.6\% \pm 1.4\%$  at 1.2 MPa,  $37.0\% \pm 11.1\%$  at 1.8 MPa,<sup>4</sup> and  $49.2\% \pm 12.2\%$  at 2.3 MPa.<sup>13</sup> This was quite similar to results in swine (Figure 2). The effect induced by intermittent scanning with the Vingmed System Five was very low at 1.3 MPa (Figure 2), but GCH was evinced by blood-filled tubules on the surface. However, the effect produced by agent-destructive bursts during contrast mode imaging with the Sequoia 512 was substantial at 1.3 MPa (Table 1, closed abdomen), possibly because of the longer pulses and burst duration. The threshold for GCH was found to be 0.73 MPa at 1.5 MHz in rats using more sensitive testing methods.<sup>4</sup>

**Figure 4.** Serial sections of glomeruli found in a kidney from group 4 with normal hematoxylin (blue) and eosin staining (A) and with hematoxylin and immunohistologic staining for fibrinogen/fibrin, which stains clots dark brown (B). The clot (C) in B extends from the glomerular capsule (G) into the neck of the first convoluted tubule (T). Scale bar indicates 50  $\mu\text{m}$ .





The relative species sensitivity depends on several factors, all of which may not be known. For example, the anaphylactoid reaction induced in pigs could reduce the circulating dose of contrast agent gas bodies and the resulting bioeffects compared to rats. The time-intensity curves obtained in the left kidney had a sham decay constant of about 26 seconds for pigs, which was much faster than the sham decay constant of about 74 seconds in rats.<sup>14</sup> Natural gas body removal by the mononuclear phagocyte system may work faster in pigs than in rats. The time-intensity curves did not, however, reveal an important loss of gas bodies through agent destruction in the exposed right kidney, which was seen in rats.

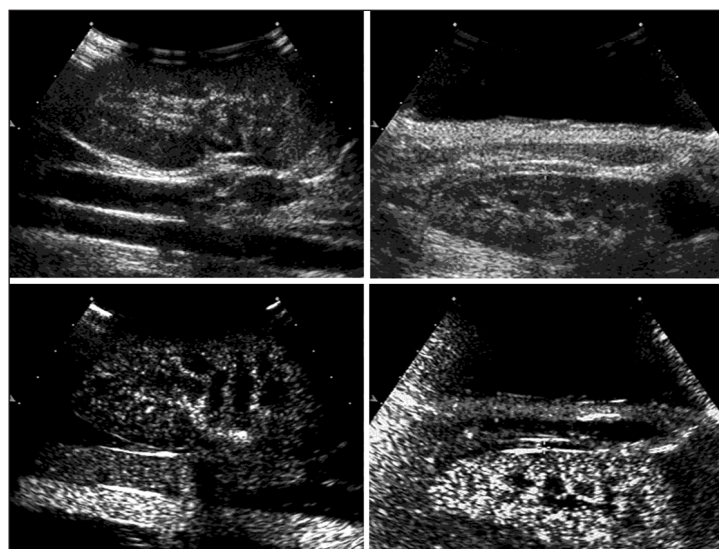
Hematuria was detected in urine samples from the swine but was not comparable to the previous results in rats<sup>5</sup> because of the very different urine collection methods. In addition, the occurrence of Bowman's space enlargement when full of blood (indicative of intratubular obstruction) and formation of clots in Bowman's space, which had been shown in rats under different conditions,<sup>6</sup> were also observed in the swine. The intratubular obstruction would be expected to cause acute tubular necrosis in effected nephrons, which had been previously seen in rats.<sup>5</sup>

**Figure 5.** Band of marks from hemorrhage into tubules on a group 6 kidney with open-abdomen scanning with the Sequoia 512 machine. Note the regular pattern of maxima in the marks with spacing of about 4 mm. Scale bar indicates 10 mm.



An interesting feature of the exposure with the Sequoia 512 was that the band of blood-filled tubule marks produced on the kidney was variable (Figure 5), with maxima in density of the marks having a regular spacing of about 4 mm. Hydrophone examination of the pulse sequences produced by the clearance burst revealed that the two patterns of pulse amplitudes shown in Figure 7 could be seen at regular intervals. The lateral spacing between the occurrence of either extreme pattern was 4.2 mm at a depth of 4 cm and varied with depth. Previously, a large difference in the GCH effect for pulse pressure-amplitude sequences that start high and decrease (ramp down) and sequences that start low and increase (ramp up) was found: the ramp-down sequence had much more effect.<sup>13</sup> The low effect for the ramp-up sequence was attributed to destruction of the optimum gas bodies before the highest PRPA pulses arrived. The ramp-down and -up sequences occur at regular intervals for the initial pulses in the agent destruction bursts shown in Figure 7. This observation explains the regular spacing of maxima in the hemorrhage-filled tubule marks shown in Figure 5 as the positions of the ramp-down sequence.

**Figure 6.** Images obtained with the Sequoia 512 machine for the probe in contact with the kidney (left) and for the probe with saline standoff and a closed abdominal wall (right). The images were obtained in the tissue harmonic mode (top row) and the contrast mode (bottom row), which shows contrast agent gas bodies in the renal circulation. The contrast mode used balance setting 1 (contrast agent only) in the bottom left image and setting 0 (contrast plus B-mode background) in the bottom right image.

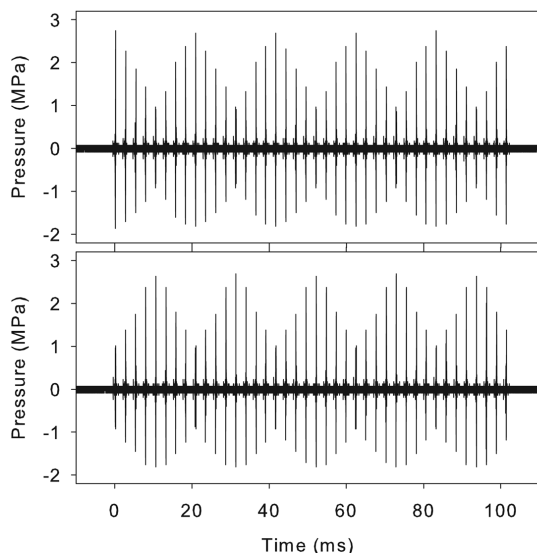


Jiménez et al<sup>7</sup> found no renal tissue damage in swine kidney after CEDUS imaging with a Sequoia 512 ultrasound machine with the probe in contact with the kidney. The depth of the maximum pulse pressure-squared integral (focal zone) was listed as 87 to 38 mm.<sup>7</sup> The reason for the lack of tissue injury appears to be the placement of the probe in contact with the kidney, which placed the focal zone outside the proximal cortex. This configuration did not produce a clear image of the proximal cortex (Figure 7). This same negative result in the proximal cortex was obtained in our study for scanning with the probe in contact with the kidney (Table 1). However, GCH was observed on the distal side of the kidney in this study, apparently because some portion of the distal cortex was within the focal zone (Figure 6). Another reason for the lack of tissue injury may have been the discrepancy in the measured PRPA for the Sequoia 512, which was much lower than expected for the displayed MI value of 1.9 (see “Materials and Methods”). The differences between Definity

and SonoVue do not seem important because both agents are suspensions of gas bodies, which are strongly activated by the diagnostic ultrasound exposure to produce enhanced image contrast. Unfortunately, there have been very few published bioeffects studies using SonoVue, so no other research data appear to be available for comparison. A study using the methods used in this study but with SonoVue would be a valuable addition to the literature on this subject.

In conclusion, renal tissue damage was produced in pigs by CEDUS. These animals were difficult subjects because of the anaphylactoid reactions to the agent and other factors. These reactions may have reduced the circulating contrast agent dose substantially, based on the wash-out time constant (see “Results”). Nevertheless, the bioeffects were substantially the same as the bioeffects seen previously in rats.<sup>4-6</sup> Glomerular capillary hemorrhage was induced and led to hematuria and intratubular obstruction in some nephrons. There was no reason to believe that similar bioeffects would not be induced in human kidneys under similar imaging-exposure conditions. The medical importance of these bioeffects is uncertain, but they are certainly undesirable. Tests for clinically observable effects, such as hematuria, should be included in clinical trials of renal CEDUS. Furthermore, the inclusion of information on ultrasound-induced bioeffects and their mitigation in contrast agent package inserts would be of value to help the sonographer ensure the optimal risk-benefit profile for the patient.

**Figure 7.** Ultrasonic pressure plots for the clearance burst of the Sequoia 512 machine in the contrast mode at 2 lateral positions about 2 mm apart in the focal zone at the same image depth of 4 cm. The pulse pressure amplitudes vary in a regular way with 5 peaks. However, note that the sequence in the top panel starts (on the left) with a maximal pulse followed by decreasing pulse heights, whereas the sequence in the bottom panel starts with a minimal pulse followed by increasing pulse heights. Sequences displayed a transition between the two extreme cases as the hydrophone was moved transversely along the scan plane.



## References

1. Averkiou M, Powers J, Skyba D, Bruce M, Jensen S. Ultrasound contrast imaging research. *Ultrasound Q* 2003; 19:27–37.
2. Miller DL, Averkiou MA, Brayman AA, et al. Bioeffects considerations for diagnostic ultrasound contrast agents. *J Ultrasound Med* 2008; 27:611–632.
3. Wible JH Jr, Galen KP, Wojdyla JK, Hughes MS, Klibanov AL, Brandenburger GH. Microbubbles induce renal hemorrhage when exposed to diagnostic ultrasound in anesthetized rats. *Ultrasound Med Biol* 2002; 28:1535–1546.
4. Miller DL, Dou C, Wiggins RC, Wharram BL, Goyal M, Williams AR. An in vivo rat model simulating imaging of human kidney by diagnostic ultrasound with gas-body contrast agent. *Ultrasound Med Biol* 2007; 33:129–135.

5. Williams AR, Wiggins RC, Wharram BL, et al. Nephron injury induced by diagnostic ultrasound imaging at high mechanical index with gas body contrast agent. *Ultrasound Med Biol* 2007; 33:1336–1344.
6. Miller DL, Dou C, Wiggins RC. Glomerular capillary hemorrhage induced in rats by diagnostic ultrasound with gas-body contrast agent produces intratubular obstruction. *Ultrasound Med Biol* 2009; 35:869–877.
7. Jiménez C, de Gracia R, Aguilera A, et al. In situ kidney insonation with microbubble contrast agents does not cause renal tissue damage in a porcine model. *J Ultrasound Med* 2008; 27:1607–1615.
8. Grauer SE, Pantely GA, Xu J, et al. Myocardial imaging with a new transpulmonary lipid-fluorocarbon echo contrast agent: experimental studies in pigs. *Am Heart J* 1996; 132:938–945.
9. Ostensen J, Hede R, Myreng Y, Ege T, Holtz E. Intravenous injection of Albunex microspheres causes thromboxane mediated pulmonary hypertension in pigs, but not in monkeys or rabbits. *Acta Physiol Scand* 1992; 144:307–315.
10. Bramos D, Tsirikos N, Kottis G, et al. The acute effect of an echo-contrast agent on right ventricular dimensions and contractility in pigs. *J Cardiovasc Pharmacol* 2008; 51:86–91.
11. Bush WH, Swanson DP. Acute reactions to intravascular contrast media: types, risk factors, recognition, and specific treatment. *AJR Am J Roentgenol* 1991; 157:1153–1161.
12. Goss JE, Chambers CE, Heupler FA Jr. Systemic anaphylactoid reactions to iodinated contrast media during cardiac catheterization procedures: guidelines for prevention, diagnosis, and treatment. Laboratory Performance Standards Committee of the Society for Cardiac Angiography and Interventions. *Cathet Cardiovasc Diagn* 1995; 34:99–104.
13. Miller DL, Dou C, Wiggins RC. Simulation of diagnostic ultrasound image pulse sequences in cavitation bioeffects research. *J Acoust Soc Am* 2007; 122:2002–2008.
14. Miller DL, Dou C, Wiggins RC. In vivo gas body efficacy for glomerular capillary hemorrhage induced by diagnostic ultrasound in rats. *IEEE Trans Biomed Eng* 2010; 57:167–174.
15. Miller DL, Dou C, Wiggins RC. Doppler mode pulse sequences mitigate glomerular capillary hemorrhage in contrast-aided diagnostic ultrasound of rat kidney. *IEEE Trans Ultrason Ferroelectr Freq Control* 2007; 54:1802–1810.