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The Dependence of Glomerular Capillary Hemorrhage Induced by Contrast Enhanced Diagnostic Ultrasound on Microbubble Diameter

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Abstract

A recently proposed two-criteria model for cavitation bioeffects in tissue with microbubbles (MBs) was tested. The glomerular capillary hemorrhage (GCH) bioeffect was observed in rat kidney for contrast agent MB suspensions with mean diameters of 1.6, 3.1 and 5.5 μm . A diagnostic ultrasound machine was used at 3.6 MHz and 5.5 MHz for intermittent scans at power settings 2 dB apart. Petechial hemorrhage (PH) counts scored on the surface of the kidneys, and glomeruli were scored in histology. Thresholds for the PH measurements were the same for the large and medium MB suspensions but substantially higher for the small MBs. For the histology, the medium MBs gave a higher threshold than the large MBs at 5.5 MHz. The pressure amplitude thresholds are in approximate agreement with theory, and the optimum MB size counterintuitively increased for increasing ultrasound frequency, as predicted. The two-criteria model of MB-associated capillary hemorrhage is supported.

Keywords

Contrast enhanced diagnostic ultrasound; Contrast microbubbles; Ultrasonic cavitation bioeffects; Mechanical Index; Theoretical microbubble dynamics

Introduction

The mechanical index (MI) is a theoretical exposure parameter for inertial cavitation in diagnostic ultrasound. The theory for the MI assumes that bubbles of all sizes relevant for cavitation nucleation exist in tissue, a condition that is approximated for ultrasound contrast agents. The MI was devised to provide science-based safety guidance for cavitation bioeffects. The MI is displayed on diagnostic ultrasound images, and is a regulated exposure

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parameter (IEC 2010; 2015). The MI is proportional to the estimated in situ peak rarefactional pressure amplitude (PRPA) in MPa, divided by the square root of ultrasonic frequency in MHz. Assuming that the threshold for bioeffects equals the inertial cavitation threshold, this dependence suggests that PRPA thresholds for cavitation bioeffects should increase as the square root of frequency. Contrarily, ultrasonic cavitation was not found to be a problem for diagnostic ultrasound (without contrast agents) owing to a lack of suitable cavitation nuclei in vivo (Carstensen et al. 2000). However, after the introduction of ultrasound contrast agents in the 1990s, the stabilized microbubbles in the agents were found to serve as ultrasonic cavitation nuclei under suitable conditions, and a range of bioeffects was reported in vitro and in vivo (Miller et al. 2008a). The MI might reasonably be expected to be the perfect safety index for contrast enhanced diagnostic ultrasound (CEDUS).

Although bioeffects of some magnitude might be expected for any level of contrast agent excitation, a reasonable benchmark bioeffect in vivo is capillary rupture, first reported by Skyba et al. (1998) and by Miller and Gies (1998). This effect can be visible to the un-aided eye, and can lead to further biologically significant consequences, such as an inflammatory response (Miller et al. 2005), or blockage of renal tubules (Miller et al. (2009). Experimental thresholds for glomerular capillary hemorrhage (GCH) in rats having CEDUS increased approximately in proportion to frequency (not its square root) from 1.5 to 7.4 MHz with proportionality constant of 0.5 MPa/MHz (Miller et al. 2008b). Recently, the threshold for capillary hemorrhage in heart tissue induced by myocardial contrast echocardiography has also been shown to be approximately proportional to frequency from 1.6 to 3.5 MHz with proportionality constant of 0.6 MPa/MHz (Miller et al. 2016). Therefore, the MI appeared to have limited utility as a CEDUS safety index, and the role of inertial cavitation threshold for this bioeffect was uncertain.

The typical cavitation criteria have been a temperature achieved upon collapse, (e. g., 5,000 K), or an expansion to twice the equilibrium diameter before collapse. However, the temperature elevation is greatly reduced for perfluorocarbon gasses, typically used in contrast agent microbubbles, and, for higher frequencies having very small optimum inertial cavitation nuclei, the doubling of diameter remains much smaller than the capillary diameter. Theoretical analysis of microbubbles in glomerular capillaries subjected to threshold diagnostic pulses was undertaken to assess the possibility that the maximum size reached during the cavitation was a key factor in capillary rupture (Patterson et al. 2012). However, calculations of maximum radius achieved for different microbubble sizes and pulses frequencies (from Miller et al. 2008b) did not reveal a consistent maximum size threshold related to the GCH thresholds (Patterson et al. 2012).

We recently proposed a two-criteria model for cavitation bioeffects in tissue with microbubbles (MBs) (Church and Miller, 2016). Cavitation thresholds were computed for linear versions of the acoustic pulses used in the kidney study (Miller et al. 2008b) by combining results for a criterion that maximized circumferential stress in the capillary wall with another that ensured an inertial collapse. This produced thresholds that were consistent with the experimental data in both threshold magnitude and frequency dependence. In addition, this model indicated that the GCH bioeffect should depend on MB diameters, and that the effective MBs should be larger than those predicted to be optimal from the MI

model. However, MBs much larger than the optimum are expected to be ineffective because these would be near resonance size, an upper limit for inertial collapse (Nyborg et al. 2002). That is, for the two criteria model, there may be a MB diameter for each frequency that is optimum (i. e. has the lowest threshold) for induction of capillary injury, and thus yields the GCH bioeffect at the thresholds.

In this study, the rat kidney GCH bioeffect was observed for contrast agent MB suspensions modified to have different mean sizes as a test of the two criteria model. A laboratory version of the contrast agent Definity, which has octafluoropropane gas-filled MBs stabilized by a lipid skin, had been produced and previously separated into two different size ranges by centrifugation methods (Miller et al. 2015). For this study, suspensions with three different size ranges were produced and tested by CEDUS at different PRPAs to find the GCH threshold for each size. An initial experiment at 3.6 MHz was somewhat inconclusive with regard to the optimum size range. The discrimination in size becomes more important at higher frequencies, and the test was repeated at 5.5 MHz. The results supported the two-criteria model relative to the MI model for cavitation bioeffects.

Methods

Animal preparation

This in vivo research was conducted with the approval of the Institutional Animal Care and Use Committee at the University of Michigan. CD hairless rats (Charles River Laboratories, Wilmington MA) were used in this study. The rats were anesthetized via intraperitoneal injection of ketamine (91 mg/kg) plus xylazine (9 mg/kg). For intravenous injections and infusion of MB contrast agent, a 24 gauge cannula was inserted into a tail vein. The anesthetized rats were mounted on a plastic holder with the left side toward the board. This mount was then placed vertically in a water bath filled with 38°C degassed water for ultrasound scanning of the right side. This method allows flexible aiming of the ultrasound probe and maintains the animal temperature. Shaving was not needed for preparation, because the rats were nearly hairless.

Diagnostic Ultrasound

The general procedure for diagnostic ultrasound exposure with contrast agent has been described previously (Miller et al. 2007). A Vivid 7 Dimension (General Electric Co., Cincinnati OH) ultrasound machine was used for scanning-exposures. Two probes were used: a 5S phased array operated at the 4 MHz setting (3.6 MHz measured center frequency), and a 7L linear array operated at the 6 MHz setting (5.5 MHz measured). The 7L probe was chosen for the tests at the higher frequency, over the 10S phased array, because no significant GCH was found with a probe similar to the 10S used at 5 MHz for a previous study (Miller et al. 2008b). Parameters for the setups and aiming of the probes are listed in Table I. Each probe was clamped in the water bath with the imaged surface of the right kidney located at the exposure depth, which was set at the position of the maximal peak rarefactional pressure amplitude (PRPA). The exposure PRPA was adjusted using the power control in 2 dB increments. At the maximum 0 dB setting, the on-screen MI value was 1.2 for both probes. The PRPA at the kidney was determined by calibrated hydrophone

measurements (HMA 0200, Onda, Sunnyvale, CA, USA) after passage through a sample of the rat abdominal wall from the position of the right kidney. These in situ exposure values were obtained at each power setting and averaged for 4 different tissue samples. The 0 dB mean pulses are shown in Fig. 1 for the two experiments with center frequencies of 3.6 MHz and 5.5 MHz, which are the key frequencies at the thresholds. The pulses are moderately broadband, with bandwidths of 2.5 and 2.8 MHz. During the exposure, the image frames were intermittently time-triggered at 0.5 Hz to allow refill of the tissue with contrast agent after MB destruction, which occurs during the higher-power imaging scans.

Ultrasound Contrast Agent

The ultrasound contrast agent was produced as needed using procedures designed to approximate the commercial agent Definity® (perflutren lipid microsphere injectable suspension, Lantheus Medical Imaging, Inc., N. Billerica, MA USA), which is no longer sold for research purposes. A replacement contrast agent (RCA) for Definity was created using a formula set as close as possible to the Definity formula, as described previously (Miller et al. 2015). To prepare the contrast medium, the required lipids were dissolved in a mixture of 80% (v/v) phosphate buffered saline, 10% (v/v) propylene glycol, and 10% (v/v) glycerol, which was heated to 70° C to solubilize the lipids and then sterile filtered with a 0.2 µm filter. The contrast medium was then aliquoted into vials with the headspace for each vial filled with octafluoropropane (HC-218, PurityPlus, Metro Welding Supply, Detroit, MI USA) and the vial was then capped and sealed. Vials either 2.8 or 3.5 ml were filled to 43% of capacity. To produce the suspensions of stabilized MBs, the vials were shaken for 45 s in a VialMix (Dupont Pharmaceuticals Co., Bellerica MA) exactly as performed for Definity. The replacement contrast agent was comparable to actual Definity, and essentially interchangeable with the clinical agent (Miller et al. 2016).

For this study, 12–15 vials were shaken to produce 18 ml of contrast MB suspension each day. The MB concentrations and size distributions were measured with a Multisizer 4 (Beckman Coulter, Inc. Indianapolis In, USA). The initial size distribution was dominated by small MBs, such that there were about 20 times more 1 µm than 5 µm MBs (Miller et al. 2016). A centrifugation method was used to obtain suspensions with different size ranges, which was based on the method of Feshitan et al. (2009). A swinging bucket centrifuge (CL3-R, International Equipment Co., Needham Heights, MA, USA) was used to apply specific relative centrifugal forces (RCF) for specific times to separate larger from smaller bubbles. The procedure is illustrated in Fig. 2. The method separates the upper MB “cake” with larger bubbles from infranatants with smaller bubbles from a suspension. By collecting large MBs using a low RCF to produce a “top cake”, resuspending it, and then removing small bubbles with a higher RCF to leave the smaller sizes in the infranatant fluid suspension, a size range is selected and relatively concentrated with several repetitions. The method employed three centrifuge stages to obtain the large, medium and small MB suspensions. The infranatant from the first, large MB stage was used as the stock for the second stage, etc., see Fig. 2.

After final resuspension, each suspension was counted and the microbubble concentrations from this complex manual procedure were variable: stock concentrations were 1.5–6 10⁸ ml

$^{-1}$ for the medium and large sizes, and $1.6\text{--}3 \times 10^9 \text{ ml}^{-1}$ for the small size. The final steps for the small MBs was often difficult due to the slow motion of the MBs in the tube, instability of the small MBs at higher RCFs, and the inability to count very small MBs; therefore, the method for the small MBs was reduced to one step to obtain sufficient MBs of the smaller sizes. All suspensions were prepared in the morning and used for exposures in the afternoon. An example of the distributions for the three sizes is shown in Fig. 3. The suspensions are distinctly different in MB size, but there is some overlap. The average diameters of the MB suspensions used in the two experiments are given in Table 2. After infusion, the distribution of sizes may change due to several processes: the lung filters out all microbubbles larger than about $8 \mu\text{m}$ diameter and diffusion of ambient gases into the microbubbles can increase the diameters, and outward diffusion reduces the sizes (Church and Miller, 2016). Calculations suggest that the magnitudes of these size changes are not sufficient to have a major influence on results, given the microbubble size distributions.

For infusion, the desired suspension was diluted by adding a volume of prepared agent to 3 ml of sterile saline to obtain $6 \times 10^7 \text{ ml}^{-1}$. The dilution was infused at 0.5 ml/kg/min , which approximately replicated the procedure for previous research using Definity (Miller et al. 2007). For exposure, the contrast infusion was started with the ultrasound on, and timed for 1 min after the contrast enhancement began to appear in the image, with scanning continued for 30 s after the infusion was stopped.

Measured Endpoints

The rats were weighed, and physiological data were collected from most rats for rectal temperature, and for heart rate and percent oxygen saturation (%SPO₂) with a pulse oximeter probe on a hind paw (SurgiVet V3395 TPR, Smiths Medical Inc. St Paul, MN USA). Glomerular capillary hemorrhage was evaluated by observation of the kidney surface and by histology, as described previously (Miller et al. 2007, 2016b). The petechial hemorrhages (PH) visible on the kidney surface were counted as a surrogate measure of relative GCH. The petechiae are urinary tubules filled with blood from the GCH in the associated glomerulus. For histology, kidney samples were removed, trimmed and immersion fixed in neutral buffered formalin. Histological processing was performed at the Research Histology and Immunoperoxidase Laboratory of the University of Michigan Comprehensive Cancer Center Tissue Core. Glomeruli were counted on three slides from the center of the scan plane including the number with any evidence of GCH, which included as few as two erythrocytes in different areas identifiable as Bowman's space. For the slide with the maximum count, the percentage of GCH was calculated. The petechiae count was the more sensitive test for GCH near the threshold, because the blood filled tubules arise from glomeruli in a much larger volume of cortex than can be seen in a histological cross section.

Experimental plan and statistics

Different groups of rats were scanned at 0 dB (maximum) power and at lower settings spaced 2 dB apart, to assess the exposure–response and find the setting with no effect. This threshold response was performed for the three MB suspensions for both probes. All groups had 6 rats except for a few tests. For the 5s probe experiment, the large 0 dB group had 5 rats, and the large -6 dB group had 7 rats. For the 7L probe experiment, the 0 dB groups for

different MB sizes all had 8 rats each. PRPA thresholds were calculated as the mean of the lowest scan PRPA with significant effect (petechiae or %GCH) and the next lowest step, which has an uncertainty of ± 1 dB or less.

Numerical results are presented as the mean plus/minus the standard deviation for the groups, or plotted as the mean with standard error bars. For statistical analysis, Mann-Whitney rank sum tests were used to compare means of the measured parameters, with statistical significance assumed at $P = 0.05$.

Results

Stereo microscope photographs of a kidney from the two experiments for each MB size are shown in Fig. 4 for the 0 dB setting. The bands of petechiae are slightly narrower for the 7L probe (5.5 MHz). The -6 dB scan plane thickness (beam width) was 3.4 mm for the 5S probe (3.6 MHz) and 1.75 mm for the 7L probe; however, the petechiae band widths are blurred due to the motion of the kidney with respiration. The PH were simply the blood field tubules visible at the surface, which were associated with a glomerulus with capillary hemorrhage. An example of a histological section is shown in Fig. 5. Some glomeruli had evidence of hemorrhage in Bowman's space which was not necessarily associated with a PH (possibly due to wash out of the erythrocytes). Nevertheless, the PH count was the more sensitive test for near-threshold exposures, since even one PH on the entire kidney was obvious on the surface (while the 5 μ m histology sections only sampled a very small part of the scan plane).

The results for PH counts and GCH scores are presented in Figures 6 and 7 for the 5S and 7L probes, respectively. The PH results were similar for large and medium MB suspensions; for example, the 0 dB results were not statistically significantly different for the 5S ($p=0.38$) and 7L ($p=0.15$) probes. However, both were significantly different from the small MB suspension results. Interestingly, the PH results at -2 dB were significantly lower for the medium MBs for both probes ($p<0.01$ and $p<0.001$ for the 5S and 7L). The histology results were also not significantly different for 0 dB, but at -2 dB were not significantly different for the 5S ($p=0.32$), but were for the 7L ($P=0.002$). This suggests that the larger MBs were more optimal than the medium MBs, especially for the higher frequency probe. Thresholds for the PH measurements are listed in Table 3. The PH thresholds for the large and medium MB suspensions were the same, while the small MB results were substantially higher by two levels (4 dB). For the histology, the thresholds were the same for large and medium MBs with the 5S probe, but the medium MBs gave a higher threshold than the large MBs for the 7L probe. Again, the larger MBs appeared more optimal for the higher frequency.

Discussion and Conclusion

This study was undertaken as an experimental test of theoretical models for the induction of GCH by CEDUS. The lowest thresholds found here were 1.6 MPa at 3.6 MHz, and 2.0 MPa at 5.5 MHz (Table 3), somewhat lower than expected. An earlier study of diagnostic ultrasound-induced GCH using Definity found that the PRPA threshold for GCH was approximately proportional to frequency from 1.5 to 7.4 MHz with a proportionality

constant of 0.5 MPa/MHz (Miller et al. 2008b), which would predict thresholds of 1.8 MPa and 2.6 MPa at 3.6 and 5.5 MHz, respectively. The higher frequency 7L probe, especially, was more effective than expected. Examining the pulse shapes (Fig. 1) reveals that, although the PRPA at 0 dB was slightly less for the 7L probe (3.5 MPa vs. 3.8 MPa for the 5S probe), the peak compressional pressure amplitude (PCPA) was substantially larger (7.9 MPa vs. 5.9 MPa). Possibly, the PCPA has a role in the collapse phase of the cavitation dynamics, although this would depend on the phase of the bubble response relative to the acoustic field.

The key MB parameter studied here for cavitation dynamics *in vivo* was the diameter. The study was performed in two separate experiments, because the first experiment did not distinguish between the medium and large sizes, whereas an optimum size was expected. The experiment was repeated at a higher frequency, to provide a more decisive test of the optimum size range. The mean MB diameters for suspensions used here were about 1.6 μm , 3.1 μm and 5.5 μm (Table 2). The smaller MBs were decidedly less effective. The effectiveness of the medium and large MB suspensions were similar but the larger diameter appeared to be slightly more effective with significantly greater PH at -2 dB. Interestingly, the larger MBs seemed to be more effective especially for the higher frequency with significantly more %GCH at -2 dB for the larger MBs, and a higher threshold for the medium MBs.

For the MI theory, which predicts a cavitation threshold dependence on the square-root of frequency, the cavitation threshold is relatively low and the optimum cavitation nuclei tend to be very small for relatively high frequencies. Using the theoretical analysis presented by Apfel and Holland (1991), the cavitation thresholds are 0.63 MPa and 0.80 MPa for 3.6 MHz and 5.5 MHz, respectively. The expected optimum sizes of nuclei are about 0.6–1.0 μm diam. The thresholds and optimum MB sizes for the GCH bioeffect are quite different in this study at 1.6–2.0 MPa and 3.0–5.5 μm diam. However, a more direct endpoint for inertial cavitation thresholds, e.g., generation of broadband noise, was not studied, so the difference in thresholds is neither surprising nor unexpected. That is, the MI theory likely is correct for inertial cavitation, but cannot be assumed to be equivalent to the bioeffect threshold.

Our recent theoretical approach utilized a two-criteria model for explaining the cavitation bioeffects thresholds (Church and Miller, 2016). This theory correctly predicts the proportionality to diagnostic ultrasound frequency and the approximate threshold PRPAs for GCH, depending on assumed physical conditions. The two-criteria model also implicates relatively large MBs in capillary injury, and the optimum size increases with increasing frequency. The equilibrium diameters for optimal GCH with 2 kPa rigidity and 5 mPa s viscosity at 3.6 MHz and 5.5 MHz were ~3.2 μm and ~4.2 μm , respectively (Church and Miller, 2016). Therefore, the interesting finding that the larger MBs were most effective at these high frequencies was predicted by the two-criteria model. This is a key prediction because other theoretical analysis, including simple microbubble resonance and the cavitation modeling for the MI, indicate that the size of the optimum microbubble should decrease with frequency. Here, The larger MBs seemed particularly effective at the *higher* frequency with more %GCH at -2 dB and lower threshold for %GCH for the larger MBs. This counter-intuitive result was predicted by the model, for which the optimum MB sizes

increase with increasing frequency (see Fig. 5, Church and Miller, 2016). These results therefore support the two-criteria model to within the range of experimental uncertainty.

It should be noted that therapeutic applications of contrast-nucleated cavitation may not follow these results for short-pulse, high frequency diagnostic ultrasound, but seem similar in some ways. McDannold et al. (2008) found that blood brain barrier disruption, intended for therapeutic purposes, followed the MI dependence from 0.26–3.0 MHz. The leakage of MRI contrast agent into the brains of rabbits was detected by magnetic resonance imaging after open skull exposures for 20 s treatment with 10 ms pulses each 1 s with Optison contrast agent. The observation of erythrocyte extravasation (i. e., capillary hemorrhage) in histology appeared to increase with increasing frequency, but a frequency dependence of that threshold was not determined. A finite-element theoretical analysis of the phenomenon for long pulses (essentially continuous wave ultrasound) at 0.55 MHz showed that intra-capillary shear stress, circumferential stress and transluminal pressure were all highest for the largest microbubbles considered, 3 μm radius (Hosseinkahah et al 2015). The calculations indicated behavior which might be considered to be stable cavitation even for pressure amplitudes exceeding the inertial cavitation threshold (MI model) of 200 kPa.

Bader and Holland (2013) evaluated theoretically the stable cavitation behavior of contrast-agent microbubbles for 110 cycle excitation of Definity, Optison and Sonazoid – model microbubbles from 0.1– 7 MHz. These parameters were relevant to the problem of cavitation-enhanced thrombolysis (sonothrombolysis). The threshold for subharmonic oscillation was taken as a criterion of potential bioeffects. This threshold was found to be generally proportional to frequency in blood, but with a low proportionality constant of 0.09 MPa/MHz, even less than that for inertial cavitation. Additionally, the optimum microbubble sizes were substantially larger for the subharmonic thresholds than for the inertial cavitation thresholds. This model does not seem to directly apply to the in vivo capillary rupture thresholds, which are much higher. Interestingly, this behavior was not unlike the analysis of in vitro cell membrane leakage induced by relatively long (36 cycle at 3.3 MHz) ultrasound pulses, with threshold of ~0.56 MPa (~0.17 MPa/MHz) (Miller and Bao, 1998). This bioeffect was correlated with subharmonic emissions, and the theoretical subharmonic threshold (for microbubbles twice the diameter for linear resonance) was approximately proportional to frequency (Miller, 1998). The stable cavitation model is therefore important for relatively low amplitude, stable excitation in therapeutic applications and also appears to have thresholds proportional to frequency. Further research to collect data on the subharmonic emissions and white noise (collapse cavitation) emissions for long-pulse exposure in vivo with contrast agent in the circulation may be informative as to the relative roles of stable and inertial cavitation in bioeffects for low-power therapy applications.

The two-criteria model for the threshold behavior of optimum contrast-agent microbubbles appears approximately to explain the frequency dependence of the capillary rupture thresholds of CEDUS in kidney. The pressure amplitude thresholds are in approximate agreement, and the optimum microbubble sizes are relatively large as predicted. Further, the optimum size appeared to actually increase for increasing ultrasound frequency, as predicted. Of course, the experimental conditions and results have uncertainty due to the MB distributions and short (wide bandwidth) pulses. Research with narrower MB distributions,

such as the mono-disperse suspensions possible with micro-fluidic methods would provide additional valuable information. In addition, more research to delineate the exact mechanism for capillary rupture would be useful for gauging CESUS risks in diagnosis and effectiveness in therapeutic applications.

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Figure 1.

The mean pulse shape at 0 dB obtained by averaging pulse shapes for passage through four rat abdominal wall samples. The center frequency of the 5S probe pulse was 3.6 MHz with a bandwidth of 2.5 MHz, while the 7L probe pulse was 5.5 MHz with a bandwidth of 2.8 MHz.

Figure 2.

A diagram of the process used to produce the suspensions with three different microbubble (MB) size ranges. After each centrifugation step at a specific relative centrifugal force (RCF) the upper cake contained the larger MBs, and the infranatant suspension had smaller MBs.

Figure 3.
Normalized microbubble size distributions for the three different suspensions.

Figure 4.
Examples of the petechial hemorrhages seen on the surface of kidneys exposed using CEDUS at 0 dB. The top row shows kidneys exposed at 3.6 MHz for small (left), medium (middle) and large (right) microbubble suspensions. The bottom row shows the results at 5.5 MHz. Scale bar: 5 mm.

Figure 5.

An example of the histological observation of glomerular capillary hemorrhage (GCH) caused by CEDUS with the 5s probe at 0 dB. The circular glomerular capsules have a Bowman's urinary space which fills with blood upon GCH, and the associated tubules often filled with blood after GCH, which were visible on the kidney surface as petechiae. Scale bars: 1 mm (left), 0.2 mm (right).

Figure 6. Results for petechiae counts and % of glomerular capillary hemorrhage for the 5S probe at 3.6 MHz for the three microbubble suspensions.

Figure 7. Results for petechiae counts and % of glomerular capillary hemorrhage for the 7L probe at 5.5 MHz for the three microbubble suspensions.

Table 1

Parameters used for operation and aiming of the diagnostic ultrasound probes.

	Frequency*	Depth	Focus	fps	prf	PD	ED
Probe	MHz	cm	cm	Hz	kHz	ns	cm
5S	3.6	8.0	5.0	31.5	3.0	390	4.2
7L	5.5	5.5	2.3	50.9	10	350	2.0

fps, frames per second; prf, pulse repetition frequency; PD, pulse duration, ED exposure depth.

* Measured center frequencies for settings of 4.0 MHz and 6.0 MHz for the 5S and 7L probes, respectively.

Table 2

Mean MB diameters with standard deviations used for the two experiments obtained by averaging the mean sizes for each exposure day.

	Small	Medium	Large
Probe	μm	μm	μm
5S	1.56 ± 0.11	3.00 ± 0.35	5.46 ± 0.40
7L	1.74 ± 0.12	3.07 ± 0.21	5.54 ± 0.32

Rat characteristics and PRPA threshold results for petechial hemorrhage counts (PH) and percentage of glomeruli with hemorrhage in histology (%GCH) for the small, medium and large MB suspensions. Uncertainty for the threshold was approximately ± 1 dB ($\pm 11\%$).

Table 3

Probe	Rat n	Weight gm	HR BPM	SpO ₂ %	PH Threshold (MPa)			%GCH Threshold (MPa)		
					Small	Med	Large	Small	Med	Large
5S	78	351 $\pm$ 45	285 $\pm$ 20	84 $\pm$ 4	2.6	1.6	1.6	3.4	2.6	2.6
7L	66	326 $\pm$ 35	292 $\pm$ 26	84 $\pm$ 3	3.1	2.0	2.0	ND	3.1	2.5

HR: heart rate in beats per minute; ND not determinable.