Lung Lesions Induced by Continuous- and Pulsed-Wave (Diagnostic) Ultrasound in Mice, Rabbits, and Pigs

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Abstract. These studies documented the presence or absence of macroscopic and microscopic intraparenchymal hemorrhage in individual lung lobes of mice, rabbits, and pigs exposed to continuous- and pulsed-wave (diagnostic) ultrasound; we described the character of and lesions associated with the hemorrhage and compared differences in the lesions among species and exposure conditions to investigate the pathogenetic mechanisms and species differences associated with ultrasound-induced lung hemorrhage. In a series of three sequential interdependent studies, 312 mice, 91 rabbits, and 74 pigs were divided at random into experimental groups and exposed to continuous-wave ultrasound (3 kHz modulated at 120 Hz) of acoustic pressure levels ranging from 0 to 490 kPa for 5, 10, or 20 minutes. In a fourth study, three mice, 43 rabbits, and six pigs were divided at random into experimental groups and exposed to pulsed-wave ultrasound (3- and 6-MHz center frequency) of peak rarefactional acoustic pressure levels ranging from 0 to 5.6 MPa for 5 minutes. Macroscopic lesions induced by continuous- and pulsed-wave ultrasound consisted of dark red to black areas of hemorrhage that extended from visceral pleural surfaces into lung parenchyma. Hemorrhage appeared spatially related to the edges of lung lobes where pleura of dorsal and ventral surfaces met, occurred in specific lung lobes in all three species, and appeared anatomically related to lung that was closest to and in contiguous alignment with the ultrasound transducer and thus the path of the sound beam. Macroscopic lesions were similar in all species under all exposure conditions for both continuous- and pulsed-wave ultrasound; however, hemorrhage was not induced in pig lung exposed to pulsed-wave ultrasound at any peak rarefactional acoustic pressure level. Eighteen mice (145 kPa exposure pressure), 60 rabbits (145–460 kPa exposure pressure), and 58 pigs (145–490 kPa exposure pressure) from study 3 were used for microscopic evaluation of lung exposed to continuous-wave ultrasound; three mice (6 MHz; 2.9 and 5.4 MPa), 39 rabbits (3 and 6 MHz; 2.3–5.4 MPa), and six pigs (3 and 6 MHz; 3.3, 5.4, and 5.6 MPa) from study 4 were used for microscopic evaluation of lung exposed to pulsed-wave ultrasound. Microscopic lesions and the character of hemorrhage induced by continuous-wave ultrasound were different from those induced by pulsed-wave ultrasound. Lesions induced by continuous-wave ultrasound under all exposure conditions were similar in all three species. Lesions induced by pulsed-wave ultrasound under all exposure conditions were similar in all three species. Microscopic lesions induced by continuous-wave ultrasound affected alveoli and alveolar septa and consisted of severe hemorrhage, accumulation of substantial volumes of protein-rich plasma admixed with fewer numbers of cells, coagulated protein, and acute coagulative necrosis. Microscopic lesions induced by pulsed-wave ultrasound consisted of severe alveolar hemorrhage without accumulation of substantial volumes of plasma, infrequent foci of coagulated plasma proteins, and absence of acute coagulative necrosis involving alveolar septa. Results of these studies suggest that 1) the principal target for the biologic effects of ultrasound in lung is the microvasculature of alveolar septa, 2) differences in the character of the hemorrhage induced by the two wave forms may reflect fundamental distinctions in physical/biological interactions that the wave forms have with cellular junctions or membranes that ultimately lead to vasolytic events and hemorrhage, and 3) innate interspecies differences in anatomy and physiology of lung may determine differences in species susceptibility to ultrasound-induced lung hemorrhage.

Key words: Continuous-wave ultrasound; hemorrhage; lung; mice; pigs; pulsed-wave ultrasound; rabbits; respiratory system; ultrasound.

Ultrasound has been widely and safely used for diagnostic and therapeutic activities in human and animal health fields for many years. Recently, safety concerns have been raised about the use of ultrasound in human medicine because severe lung hemorrhage and death were produced in mice following exposure to ultrasound at acoustic pressure levels comparable to those used in diagnostic applications and in studies demonstrating that various treatment applications of ultrasound can induce lung hemorrhage in animals.[4,6,10] Even though lung hemorrhage can be produced in mice at diagnostic ultrasound acoustic pressure levels, there

are no reports of lung injury following the use of ultrasound in human or animal medicine. Because important differences[12,13] exist, for example, in alveolar volume, diameter, and size, lung compliance, and pleural thickness among mice, rabbits, pigs, and human beings,[11,15,18,20,22–24] extrapolation of results of mouse studies and probably of rabbit studies to human safety concerns may be inappropriate.

We recently completed a series of four sequential interdependent studies that tested the hypothesis that significant species differences exist in responses of lung to ultrasound.[12–13] Allometry of respiratory variables plotted against anatomic/physiologic features has documented the placement of and positional relationships among most animal species.[20] Mice, rabbits, and pigs were selected for these studies on the basis of these allometric relationships. Pigs more closely resembled human beings, mice were far removed from human beings, and rabbits were ranked between mice and pigs. Our studies demonstrated that mice were extremely sensitive to the effects of continuous- and pulsed-wave ultrasound.[12,13] One hundred percent of mice died of massive intraparenchymal lung hemorrhage during a 10-minute exposure to 30-kHz continuous-wave ultrasound at 145 kPa.[13] No rabbits died during a 10-minute exposure to 30-kHz continuous-wave ultrasound at 460 kPa; however, mild focal lung hemorrhage was observed (W. D. O'Brien, Jr. and J. F. Zachary, in preparation).[12] No pigs died during a 10-minute exposure to 30-kHz continuous-wave ultrasound at 490 kPa; however, minimal focal lung hemorrhage was observed (W. D. O'Brien, Jr. and J. F. Zachary, in preparation). Hemorrhage also was produced in lungs of rabbits during a 10-minute exposure to 30-kHz continuous-wave ultrasound at 145 kPa (same exposure conditions that killed mice); minimal focal hemorrhage was produced in the lungs of a small number of pigs during a 10-minute exposure to 30-kHz continuous-wave ultrasound at 145 kPa and at 460 kPa (same exposure conditions that caused mild hemorrhage in rabbits). Similar species differences were observed in mice and rabbits in response to pulsed-wave ultrasound (W. D. O'Brien, Jr. and J. F. Zachary, in preparation). Pulsed-wave ultrasound did not cause hemorrhage in pig lung (W. D. O'Brien, Jr. and J. F. Zachary, in preparation). Species differences in lung responses to ultrasound determined in our studies paralleled the positional relationships of these species on allometric analyses of respiratory variables plotted against anatomic/physiologic features.[20] Mice appear to be an effective model for studying ultrasound-induced lung hemorrhage but are probably not an appropriate model to extrapolate or estimate potential lung damage in other species.[12,13]

The purpose of this study was to 1) document the macroscopic and microscopic lesions produced by continuous- and pulsed-wave ultrasound in mice, rabbits, and pigs; 2) determine if similar lesions were produced in all species under different exposure conditions; and 3) relate the lesions to bioacoustic principles that could explain the genesis of the lesions mechanistically. Additional manuscripts published from these studies have documented the qualitative and quantitative assessment, statistical analysis, and comparative evaluation of data collected from all three species under the described exposure conditions (W. D. O'Brien, Jr. and J. F. Zachary, in preparation).[12,13]

Materials and Methods

Animals

Six- to 7-week-old (30–40 g) C3H male mice obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, IN) were sonicated within 1 week of receipt. Five- to 5.5-month-old (3.6–4 kg) New Zealand White male rabbits obtained from Myrtle's Rabbitry (Thompson Station, TN) were sonicated within 1 week of receipt. Ten- to 12-week-old (27.2–31.8 kg) crossbred pigs obtained from the swine breeding unit at the University of Illinois (Urbana, IL) were sonicated within 2 days of receipt. Animals were obtained from vendors with a known history of providing healthy animals for research studies. Animals were observed by visual inspection to be free of clinical signs suggestive of respiratory disease before the start of the studies and were confirmed to be free of respiratory disease at postmortem examination.

Animals were provided housing, food, and veterinary care according to University of Illinois and NIH guidelines. Animals were anesthetized and euthanatized by methods approved by the American Veterinary Medical Association, University of Illinois Office of Laboratory Animal Care, and University of Illinois Animal Care Committees. Mice were anesthetized with ketamine hydrochloride (125.0 mg/kg) and xylazine (25.0 mg/kg) administered intraperitoneally. Rabbits were anesthetized with ketamine hydrochloride (35.0 mg/kg) and xylazine (5.0 mg/kg) administered subcutaneously. Pigs were anesthetized with ketamine hydrochloride (5.0 mg/kg), xylazine (5.0 mg/kg), and Telazol® (10 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) administered intramuscularly.

Ultrasound design, exposure, and calibration

Table 1 summarizes the 30-kHz continuous-wave ultrasound exposure conditions and number of animals of each species for studies 1, 2, and 3; Table 2 summarizes the pulsed-wave ultrasound exposure conditions and number of animals of each species for study 4. The basic differences between continuous- and pulsed-wave ultrasound are 1) two orders of magnitude difference in frequency, 2) broad beam at low frequency versus focused beam at high frequency, and 3) a factor of ≥4 difference in acoustic pressure levels.

A commercial therapeutic product for human beings (HydroSound®, Swen Sonic, a Division of Arjo Hospital Equipment, Inc., Morton Grove, IL) uses whole body exposure at 30-kHz continuous-wave ultrasound that results in 50–100 kPa maximum output acoustic pressure levels.

Table 1. Number of animals in each group and summary of 30-kHz continuous-wave exposure conditions (minutes) at various hydrophone zero-to-peak acoustic pressures (p_{hop}) for studies 1–3.

| p_{hop} (kPa) | Study 1—Mice | | | Study 2—10 Minutes | | Study 3—10 Minutes | | |
	5 Minutes	10 Minutes	20 Minutes	Mice	Rabbits	Mice	Rabbits	Pigs
0	15	15	15	4	2		15	16
65	15	15	15					
80	15	15	15					
87	15	15	15					
100	15	15	15	10	7			
145	15	15	15	10	7	18	15	16
290							16	16
340							15	
460							14	17
490								9
Total	90	90	90	24	16	18	75	74

Table 2. Number of animals in each group and summary of 3- and 6-MHz pulsed-wave exposure conditions (minutes) and derated peak rarefactional pressure ($p_{r.3}$) at various derated spatial peak pulse average intensities (I_{SPPAd}) for study 4.

| I_{SPPAd} (W/cm²) | 3 MHz, 5 Minutes | | | | 6 MHz, 5 Minutes | | | |
| | $p_{r.3}$ (MPa) | No. Animals | | | $p_{r.3}$ (MPa) | No. Animals | | |
		Mice	Rabbits	Pigs		Mice	Rabbits	Pigs
0			4*					
200	2.3		5		2.0		5	
300	2.6		2					
420	3.3		9					
480	3.3			1				
510	3.3			1	2.9	2	10	
530	3.3			1				
1,060					4.7		9	
1,310					5.4	1	3	2
1,480					5.6			1
Totals		0	20	3		3	27	3

* Sham at $I_{SPPAd} = 0$ is independent of frequency.

Our studies examined the effects of continuous (30 kHz, 0–490 kPa) ultrasound at acoustic pressure levels greater than those used for therapeutic purposes on human beings.

The maximum output levels of pulsed-wave ultrasound used in commercial diagnostic ultrasound (Advanced Technology Laboratories HDI® ultrasound imaging system, Bothell, WA) have been established by the Food and Drug Administration (FDA, Rockville, MD).[7,8] Pulsed-wave (3 and 6 MHz, 0–5.6 MPa) ultrasound levels used at all of the 3-MHz pulsed acoustic pressure levels were within the permitted FDA limits for use on human beings, with the highest acoustic pressure levels being just at the maximum levels permitted. For the 6-MHz pulsed acoustic pressure levels, the highest two levels (5.4 and 5.6 MPa) exceeded the maximum levels permitted for use on human beings, and the remaining pressure levels were within the exposure range permitted by the FDA.[1,7,8]

The data reported here were collected from four sequential interdependent studies. The basic experimental protocols were the same for studies 1, 2, and 3, which were conducted at an ultrasonic frequency of 30 kHz (continuous wave) but were different from those of study 4, which was conducted at center frequencies of 3 and 6 MHz (pulsed wave). Results of studies 1 and 2 have been published;[12,13] results of studies 3 and 4 are being assembled and manuscripts will be submitted for review in the near future. Different acoustic pressure levels were applied to different animal species based on specific experimental protocols. The goal of study 1 was to determine exposure-effect responses and to assess threshold damage levels in mice at three exposure durations. The highest acoustic pressure level (145 kPa) was determined prior to onset of the actual study; this level killed all mice at all three exposure durations. The goal of study 2 was to assess relative sensitivities in two different animal species, and the exposure conditions selected were based on findings from study 1. The goals for study 3 were the same as those for studies 1 and 2; exposure-effect responses and relative sensitivities were examined in two additional species (rabbits and pigs). The

lowest acoustic pressure level of 145 kPa for a 10-minute exposure duration was used in study 3 to have a basis for comparison of all three species at identical exposure conditions. Acoustic pressure levels higher than 145 kPa in study 3 were required to produce adequate exposure-effect responses, because rabbits and pigs were much less sensitive than mice. The highest acoustic pressure level used in these studies was the maximum output achievable by the equipment.

The goal of study 4 was to determine exposure-effect responses in rabbits at two ultrasound frequencies of 3 and 6 MHz (mice were used as positive controls and pigs were used to evaluate whether an effect could be produced at acoustic pressure levels known to produce lung damage in rabbits). Prior to the onset of the actual study, the acoustic pressure level that induced minimal hemorrhage in rabbit lung was determined. The highest acoustic pressure level used in this study was the maximum output achievable by the equipment.

For each anesthetized animal in studies 1, 2, and 3, the skin surrounding the rib cage, sternum, and vertebral column was clipped with an electric shaver and the hair was removed with a commercial depilatory agent (Nair®, Carter Products, New York, NY) to minimize the likelihood of entrapment of air at the skin–water interface. The area clipped and depilated included skin of the rib cage, sternum, and vertebral column in a circumferential plane around a cranial–caudal plane originating from a dorsal–ventral plane through the thoracic inlet and ending at a dorsal–ventral plane through the umbilicus. The anesthetized animal was restrained in a spread-eagle position, which permitted its placement vertically in the waterbath (distilled, degassed water). The ventral surface of the animal was positioned toward the ultrasound source. The individual preparing each animal for sonication (anesthetizing, depilating, and placing in exposure structure)

was blind to the exposure condition. During exposure periods, all animals were observed for alterations in respiratory rate and breathing patterns.

The exposure procedures were identical for studies 1, 2, and 3 with the exception that the temperature of the waterbath was at 37 C for study 1 and at room temperature (22 C) for studies 2 and 3. This temperature difference was necessary because studies 2 and 3 required a much larger volume of distilled, degassed water and it was not feasible to use heated water. Mice, rabbits, and pigs were exposed to acoustic pressure (defined as the total pressure minus the ambient pressure[1,2]) using a rectangular (16 × 11 cm) magnetostrictive source driven by a Blue Wave amplifier (Swen Sonic) at a frequency of 30 kHz (acoustic wavelength in water was 5 cm) modulated at 120 Hz (100% modulation factor). There was no measurable difference in the calibrated Naval Research Laboratory (NRL) Model 42D/115 hydrophone's response at the two different temperatures of the waterbaths. The NRL hydrophone sensitivity was, typically, 37.6 kPa/V. From this calibration value (supplied by NRL with the hydrophone), the hydrophone's zero-to-peak voltage (V_{hop}) was converted into the hydrophone's zero-to-peak acoustic pressure (p_{hop}) by the expression:

$$p_{hop} \text{ (kPa)} = \text{hydrophone's sensitivity (kPa/V)} \times V_{hop} \text{ (V)}.$$

The NRL hydrophone was connected in parallel to a Tektronix 466 storage oscilloscope to record the hydrophone's V_{hop}.

For the 30-kHz (continuous-wave) studies (Table 1), the acoustic pressure lateral distribution (active rectangular source area was 16 cm horizontally × 11 cm vertically) was not very uniform at a range of 5 cm from the source surface, the location of the animal's sternum. The approximate dimensions of the surface area of exposure for a mouse's thorax (sternal area) was about 2 × 2 cm, for a rabbit's thorax was about 10 × 10 cm, and for a pig's thorax was about 22 × 30 cm. To ensure that each animal was positioned at the same field location where the acoustic pressure was known, a removable metal pointer that could be placed on the face of the transducer was designed and fabricated. The acoustic pressure calibrations and animal placements were performed relative to the position of the tip of the removable metal pointer. The tip was located at a distance of 5 cm from the face of the source. The lateral area chosen to perform the calibrations, and hence the animal exposures, was a 4-cm^2 region surrounding the pointer's tip location for mice and a 100-cm^2 region surrounding the pointer's tip location for rabbits. A two-dimensional lateral beam plot at a range of 5 cm was performed (20 × 20 cm in 0.5-cm steps). Within the mouse's 4-cm^2 square region, the hydrophone's p_{hop} ranged from 99 to 118 kPa and varied by 19 kPa. Within the rabbit's 100-cm^2 square region, the hydrophone's p_{hop} ranged from 23 to 118 kPa and varied by 95 kPa. For the pig exposure conditions, the pig's thoracic area (about 22 cm horizontally × 30 cm vertically) was greater than the source's area (11 × 16 cm). Acoustic pressure levels at 30 kHz were reported with an uncertainty of ±20% (W. D. O'Brien, Jr. and J. F. Zachary, in preparation).[12,13]

For each anesthetized animal in study 4 (pulsed-wave ultrasound), the skin on the left lateral side was clipped with

an electric shaver and the hair removed with a depilatory agent (Nair®) to maximize sound transmission. The anesthetized animal was placed on its right side with the left lateral side upward. The left lateral side of the animal was in direct contact (using a commercial coupling agent) with the ultrasound transducer. The transducer was firmly supported by clamps connected to a solid supporting structure and was directed between the ribs (intercostal space) so the beam was focused over the entire left lobe (mice) or the left caudal lobe (rabbits and pigs). Verification that the ultrasound beam was directed towards lung was based on evaluation of the ultrasound image with the system operating at very low acoustic pressure levels. The individual preparing each animal for sonication (anesthetizing, depilating, and placing in exposure structure) was blind to the exact exposure condition. During exposure periods, all animals were observed for alterations in respiratory rate and breathing patterns.

Animals in study 4 were exposed to ultrasound using an Advanced Technology Laboratories HDI® ultrasound imaging system and one of two transducers (ATL Model P3.5-28 at 3 MHz and ATL Model L10-5 at 6 MHz). The ultrasound fields were calibrated by the manufacturer prior to the study. Following the 2-day study, the HDI® system was calibrated at the Bioacoustics Research Laboratory (BRL) at the University of Illinois within 24 hours after the completion of the study and then again by the manufacturer. All calibrations were performed in accordance with a national standard,[1,2] and the results (Table 2) of the pulsed-wave fields were reported in terms of their derated spatial peak pulse average intensity ($I_{SPPA d}$) and derated peak rarefactional pressure ($p_{r.3}$). Peak rarefactional pressure levels at 3 and 6 MHz were reported with an uncertainty of ±15% (W. D. O'Brien, Jr. and J. F. Zachary, in preparation).

Macroscopic evaluation

All animals were euthanatized while under anesthesia. Mice were euthanatized by exsanguination and decapitation; rabbits and pigs were euthanatized by CO_2 and exsanguination. As directed by the research sponsor, pigs in the pulsed-wave ultrasound study (study 4) were euthanatized by an overdose of barbiturate and exsanguination. Lungs were handled gently, dissected free from the thoracic cavity, and evaluated. Evaluation of mouse and rabbit lungs included examination with a dissecting microscope. Rabbit and pig lungs were sectioned in serial transverse sections to identify areas of hemorrhage in all lobes. For all animals, areas of hemorrhage were recorded in a semiquantitative manner by drawing areas of hemorrhage (in relative proportion to lungs size) on diagrams representing dorsal and ventral views of all lung lobes.[5,16] In preliminary studies, serial sections of sonicated lungs were examined macroscopically and microscopically to verify that the macroscopic interpretation of lung lesions as hemorrhage was, in fact, accurate.

Microscopic evaluation

Animals were necropsied and lung specimens were selected and placed in fixative within 10 minutes after death. The entire lung (mouse) or representative lung lesions and asso-

Table 3. Evaluation and comparison of microscopic lung lesions induced by continuous-wave (30 kHz) and pulsed-wave ultrasound in studies 3 and 4.

Wave Form	n	Number with Lesions	Necrosis	Hemorrhage Plasma > Cells	Hemorrhage Cells > Plasma	Contiguous with Pleura
Continuous						
Mice*	18	18	16	18	0	18
Rabbits†	60	58	42	54	4	58
Pigs‡	58	40	17	26	14	40
Pulsed						
Mice	3	3	0	0	3	3
Rabbits§	39	18	0	0	18	18
Pigs	6	0	0	0	0	0

* Six mice (0 kPa exposure group) from study 1 were used as controls.

† Fifteen rabbits (0 kPa exposure group) from study 3 were used as controls.

‡ Sixteen pigs (0 kPa exposure group) from study 3 were used as controls.

§ Four rabbits (0 MPa exposure group) from study 4 were used as controls.

ciated normal lung tissue (rabbit and pig) were fixed by immersion in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). Mouse lung was processed in toto, and the entire lung was sectioned in a longitudinal plane for microscopic evaluation; representative samples of rabbit and pig lung were examined. From study 3, 18 mice (145 kPa exposure pressure), 60 rabbits (145–460 kPa exposure pressure), and 58 pigs (145–490 kPa exposure pressure) were used for microscopic evaluation of lung exposed to continuous-wave ultrasound (Table 3). From study 4, three mice (6 MHz; 2.9 and 5.4 MPa), 39 rabbits (3 and 6 MHz; 2.3–5.4 MPa), and six pigs (3 and 6 MHz; 3.3, 5.4, and 5.6 MPa) were used for microscopic evaluation of lung exposed to pulsed-wave ultrasound (Table 3). Lungs from all control animals in studies 3 and 4 (Tables 1 and 2) were examined microscopically. In study 3 (continuous wave), control lung for evaluation was selected from areas that were known to have visible hemorrhage in exposed animals. In study 4 (pulsed wave), control lung for evaluation was selected from areas that were known to have visible hemorrhage in exposed animals (area where sound beam was focused on left lung). Lungs from the six pigs exposed to pulsed-wave ultrasound did not have macroscopic hemorrhage, but lung tissue specimens sampled from areas where the sound beam was focused on the left lung were examined microscopically.

Because of defined experimental protocols, number of experimental animals in each study, volume of fixative required, and biohazards associated with the handling and disposal of the fixative and to minimize any alteration of the distribution of alveolar hemorrhage and the character (qualitative ratio of plasma to cells) of the hemorrhage, lung was not fixed by instillation of formalin into the trachea. In each

Table 4. Determination of individual lung lobe sensitivity to 30-kHz continuous-wave ultrasound exposure in mice pooled from studies 1, 2, and 3. Each surface (dorsal and ventral) of five individual lung lobes was evaluated for the presence or absence of intraparenchymal hemorrhage, and the percent and number of affected lobes were calculated from a total of 162 mice that had hemorrhage in one or more of the individual lung lobes.

Lung Lobes	Percent Affected Lobes	Number Affected Lobes
Dorsal view		
Left	39.5	64
Postcaval	68.5	111
Right cranial	37.0	60
Right middle	89.5	145
Right caudal	33.3	54
Ventral view		
Left	49.4	80
Postcaval	69.1	112
Right cranial	30.3	49
Right middle	90.7	147
Right caudal	43.2	70

study, the method of euthanasia combined with immersion fixation provided excellent preservation of morphologic features of cells and septa as well as adequate distention of alveoli for histologic evaluation.

In each animal selected for microscopic evaluation, all areas of hemorrhage were evaluated microscopically. Data collected included the presence or absence of hemorrhage and necrosis, the character of the hemorrhage (qualitative ratio of plasma to cells), and the relationship between the location of hemorrhage and the visceral pleura. The character of hemorrhage was placed into one of two qualitative categories: 1) hemorrhage consisting predominately of protein-rich plasma admixed with fewer numbers of cells or 2) hemorrhage consisting predominately of cells admixed with smaller quantities of plasma. The relationship between the location of hemorrhage and its association with visceral pleura was evaluated to determine if the hemorrhage was contiguous with a pleural surface and to assess if foci of hemorrhage were present in lung parenchyma unrelated to pleural surfaces or areas of hemorrhage contiguous with pleural surfaces. Areas of lung hemorrhage unrelated to pleural surfaces and the focus of the sound beam have been reported recently in monkeys exposed to pulsed-wave ultrasound.[19]

Results

Clinical findings

These findings have been reported previously.[12-13] Clinical signs were observed only in mice exposed to continuous-wave ultrasound. Mice exposed to moderate pressure levels had elevated respiratory rates; mice exposed to higher pressure levels (100 kPa or 145 kPa) initially had elevated respiratory rates followed by a slowed gasping respiratory pattern and death.

Table 5. Determination of individual lung lobe sensitivity to 30-kHz continuous-wave ultrasound exposure in rabbits pooled from studies 2 and 3. Each surface (dorsal and ventral) of seven individual lung lobes was evaluated for the presence or absence of intraparenchymal hemorrhage, and the percent and number of affected lobes were calculated from a total of 59 rabbits that had hemorrhage in one or more of the individual lung lobes.

Lung Lobes	Percent Affected Lobes	Number Affected Lobes
Dorsal view		
Left cranial cranial	23.7	14
Left cranial caudal	86.4	51
Left caudal	89.8	53
Postcaval	37.3	22
Right cranial	39.0	23
Right middle	69.5	41
Right caudal	27.1	16
Ventral view		
Left cranial cranial	22.0	13
Left cranial caudal	84.7	50
Left caudal	88.1	52
Postcaval	37.3	22
Right cranial	39.0	23
Right middle	71.2	42
Right caudal	27.1	16

Table 6. Determination of individual lung lobe sensitivity to 30-kHz continuous-wave ultrasound exposure in pigs from study 3. Each surface (dorsal and ventral) of seven individual lung lobes was evaluated for the presence or absence of intraparenchymal hemorrhage, and the percent and number of affected lobes were calculated from a total of 49 pigs that had hemorrhage in one or more of the individual lung lobes.

Lung Lobes	Percent Affected Lobes	Number Affected Lobes
Dorsal view		
Left cranial cranial	26.5	13
Left cranial caudal	67.4	33
Left caudal	20.4	10
Postcaval	18.4	9
Right cranial	32.7	16
Right middle	57.1	28
Right caudal	14.3	7
Ventral view		
Left cranial cranial	26.5	13
Left cranial caudal	67.4	33
Left caudal	36.7	18
Postcaval	18.4	9
Right cranial	28.6	14
Right middle	53.1	26
Right caudal	14.3	7

Macroscopic findings

Continuous-wave ultrasound produced macroscopic hemorrhage in lungs of mice, rabbits, and pigs. In mice, hemorrhage occurred in all lung lobes, but the predominate target lobes were the right middle and postcaval (Table 4, Fig. 1). In addition, mice exposed at high acoustic pressure levels (100 kPa and 145 kPa) had substantial quantities of blood mixed with air bubbles in the thoracic cavity. This blood originated from visible lacerations in visceral pleura associated with areas of severe intraparenchymal hemorrhage.[12,13] In rabbits, hemorrhage occurred in all lung lobes, but the predominate target lobes were the caudal portion of the left cranial, the left caudal, and the right middle (Table 5, Fig. 2). Rabbits had no hemothorax. In pigs, hemorrhage occurred in all lung lobes, but the predominate target lobes were the caudal portion of the left cranial and the right middle (Table 6, Fig. 3). Pigs had no hemothorax. In all species, macroscopic hemorrhage was always associated with pleural surfaces at the edges of the lobe, at the edges of lungs where fissures separated lung lobes, and at the edges of lungs where they anatomically opposed the sternum and costal–chondral articulations of the sternum with the ribs.

Pulsed-wave ultrasound produced macroscopic hemorrhage in lungs of mice and rabbits and no hem-

orrhage in lungs of pigs. In mice, hemorrhage occurred in the pleura and subjacent lung of several lobes following exposure; in rabbits, hemorrhage generally occurred in pleura and subjacent lung (left caudal lobe) that was contiguous with the ultrasound beam originating from the overlying transducer head; in pigs, pulsed-wave ultrasound did not produce lung hemorrhage (Table 3). In several rabbits, foci of lung hemorrhage were observed in association with pleural surfaces of ipsilateral and contralateral lung far removed from the point of transducer contact with the skin. A similar finding has recently been reported in monkeys exposed to pulsed-wave ultrasound.[19]

Microscopic findings

Lesions and the character of hemorrhage (relative ratio of plasma to cells) differed between lungs exposed to continuous-wave ultrasound and lungs exposed to pulsed-wave ultrasound. Lesions and the character of the hemorrhage in lungs of mice, rabbits, and pigs were similar in all animals exposed to continuous-wave ultrasound. Lesions and the character of the hemorrhage in lungs of mice, rabbits, and pigs were similar in all animals exposed to pulsed-wave ultrasound. Lesions in animals exposed to continuous-wave ultrasound consisted of alveolar hemorrhage composed predominately of substantial quantities of protein-rich plasma

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2

Fig. 1. Lung, dorsal view; mouse. Representative lung lesions following exposure to 30-kHz continuous-wave ultrasound at 145 kPa for 10 minutes. Extensive dark red to red-black areas of hemorrhage (arrowheads) were most commonly observed in right middle and post-caval lobes.

Fig. 2. Lung, dorsal view; rabbit. Representative lung lesions following exposure to 30-kHz continuous-wave ultrasound at 290 kPa for 10 minutes. Small focal dark red to red-black ares of hemorrhage (arrowheads) associated with the edges of lung lobes were most commonly observed in the caudal portion of the left cranial lobe, the left caudal lobe, and the right middle lobe.

admixed with fewer numbers of cells (Table 3, Fig. 4). In some animals, cells were a substantial component of the hemorrhage; however, the plasma component was still apparent. Plasma stained intensely with eosin in many areas, suggesting a high protein concentration; in other areas this protein had a more fibrillar (possible fibrinogenesis) to coagulated appearance. Occasionally rabbits exposed to continuous-wave ultrasound had fibrin thrombi in blood vessels. Areas of acute coagulative necrosis affecting alveolar septa and terminal airways, contiguous with visceral pleura that was also necrotic, were associated with areas of hemorrhage (Fig. 5). Necrosis was observed most frequently in the tips of lung lobes where the hemorrhage appeared to originate and extended into proximal regions of the lobe.

Lesions in animals exposed to pulsed-wave ultrasound consisted of alveolar hemorrhage composed predominately of cells admixed with low to scant quantities of visible plasma and occasional foci of plasma with a fibrillar appearance (Table 3, Fig. 6). Plasma that was visible stained poorly with eosin, suggesting a low protein concentration. Acute coagulative necrosis of alveolar septa and terminal airways was not observed in animals exposed to pulsed-wave ultrasound.

With either wave form (continuous or pulsed), there were no lesions in bronchi or bronchioles, in the macrovasculature, or in capillaries in connective tissue surrounding bronchioles. In areas where alveolar septal necrosis was pronounced, associated small to medium-sized vessels and airways also were necrotic.

Discussion

Microscopic evaluation of lung exposed to continuous- or pulsed-wave ultrasound failed to demonstrate lesions in the macrovasculature that could explain hemorrhage in mice, rabbits, or pigs. This finding and the presence of hemorrhage in association with acute coagulative necrosis in animals exposed to continuous-wave ultrasound suggested that hemorrhage arose from injury to alveolar septa, specifically the microvasculature. This injury could result from simple physical or mechanical trauma (laceration or tearing) caused by the energy of the acoustic pressure wave being transferred through lung (deformation response) or by more complicated mechanisms related to cavitation-like phenomena.[3,21]

Physical injury such as tearing or laceration may be associated with direct mechanical effects of ultrasound
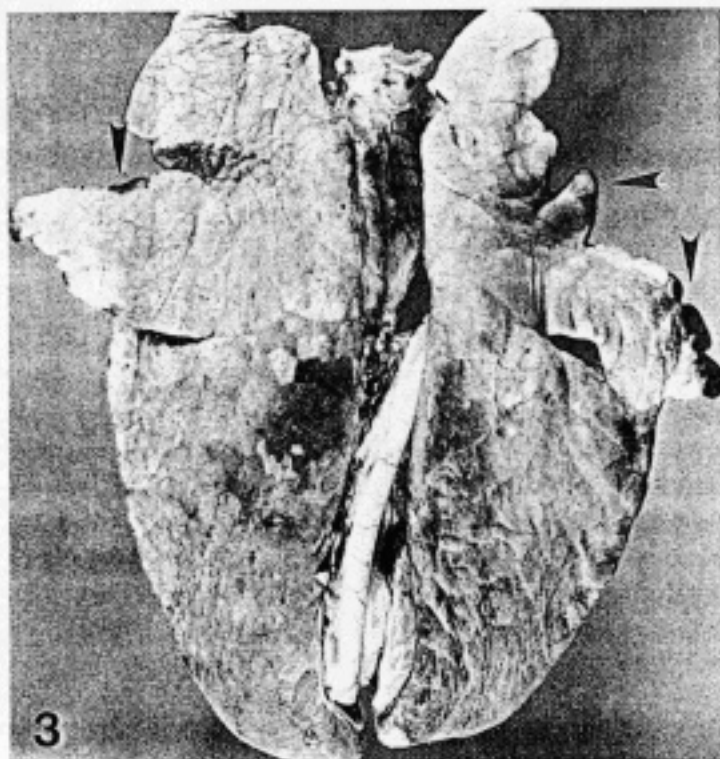
Fig. 3. Lung, dorsal view; pig. Representative lung lesions following exposure to 30-kHz continuous-wave ultrasound at 460 kPa for 10 minutes. Minimal focal dark red to red-black areas of hemorrhage (arrowheads) associated with the edges of lung lobes were most commonly observed in the caudal portion of the left cranial lobe and the right middle lobe.

on pleural surfaces and alveolar septa. This hypothesis is supported by the observation that hemorrhage occurs as early as 1 minute after exposure to continuous-wave ultrasound (J. F. Zachary, unpublished data).

Species differences in responses to ultrasound may be a reflection of structural, functional, and physiological differences in innate mechanical properties,[12,13] such as alveolar surface area, diameter, or volume, thickness of alveolar septa, lung compliance, and pleural thickness.[11,15,18,20,22-24] Tissue attenuation between the skin and pleural surfaces is unlikely to play a role in determining a species sensitivity to ultrasound of either wave form. For the continuous-wave studies at 30 kHz (studies 1, 2, and 3), the estimated attenuation coefficient is about 0.03 dB/cm. The mean, maximum, and minimum distances between skin and pleural surfaces for rabbits and pigs are 1.32, 1.60, and 0.96 cm and 2.10, 2.42, and 1.90 cm, respectively. Thus, for the maximum path lengths of 1.60 cm and 2.42 cm, respectively, for rabbits and pigs, there would be a 0.5% or 0.8%, respectively, reduction in the acoustic pressure between the skin and pleural surface, values well within the experimental uncertainty for the measurement of acoustic pressure.

For the pulsed-wave experiments at 3 and 6 MHz (study 4), the exposure acoustic pressure levels are reported as the derated values at the site of the pleural surface, where the derating factor is assumed to be 0.3 dB/cm-MHz.[1,7,8] However, this value may be an overestimation of the acoustic pressure levels at the pleural surface because the actual attenuation coefficient is >0.3 dB/cm-MHz and probably more likely (although there are no known measurements) to be approximately 1 dB/cm-MHz. Using a derating factor of 1 dB/cm-MHz and the mean thickness between the skin and pleural surfaces of the rabbit of 1.32 cm, the 3- and 6-MHz acoustic pressure levels at the pleural surface are es-
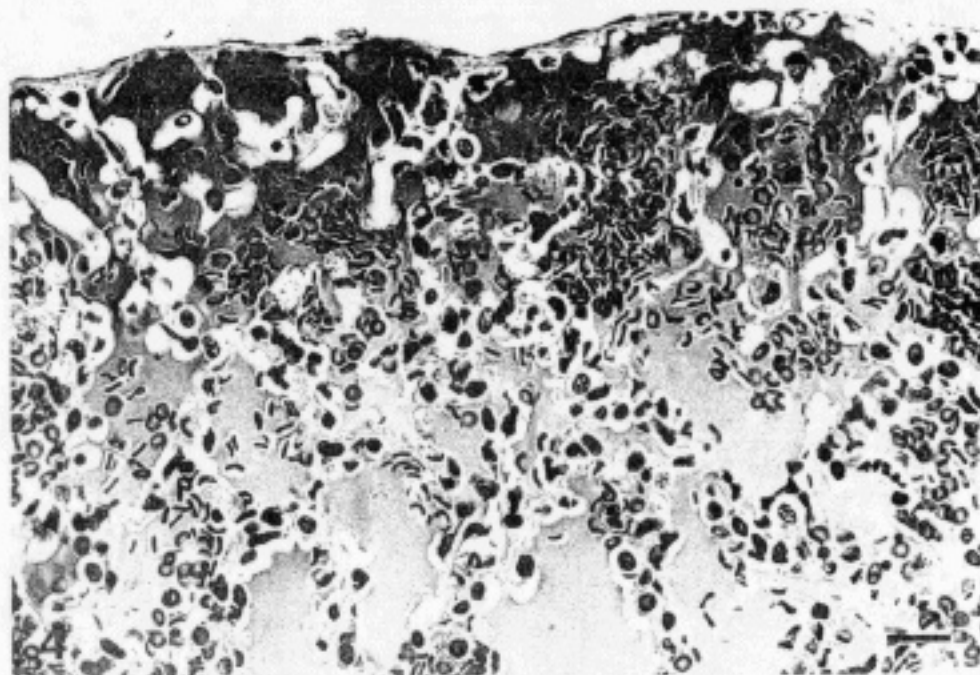

Fig. 4. Lung; rabbit. Lung was exposed to 30-kHz continuous-wave ultrasound at 460 kPA for 10 minutes. Alveolar hemorrhage consisted of protein-rich plasma admixed with fewer numbers of cells. Similar lesions were observed in mouse and pig lung following exposure to continuous-wave ultrasound. HE. Bar = 20 μm.
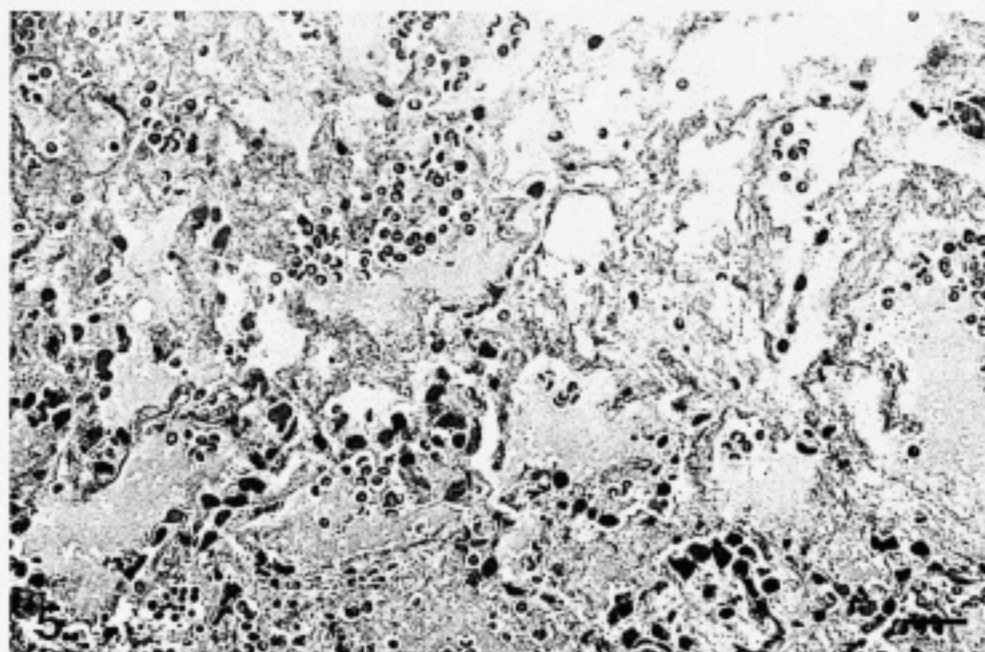
Fig. 5. Lung; mouse. Lung was exposed to 30-kHz continuous-wave ultrasound at 145 kPA for 10 minutes. Acute coagulative necrosis of alveolar septa was evident. Similar lesions were observed in rabbit and pig lung exposed to continuous-wave ultrasound but were not observed in lung of any species following exposure to pulsed-wave ultrasound. HE. Bar = 20 μm.

timated to be 73% and 53% that of the reported $p_{r.3}$ derated acoustic pressure values, respectively. For pigs, using a derating factor of 1 dB/cm-MHz and the mean thickness between the skin and pleural surfaces of 2.10 cm, the 3- and 6-MHz acoustic pressure levels at the pleural surface are estimated to be 60% and 36% those of the reported $p_{r.3}$ derated acoustic pressure values, respectively. It is appropriate to report acoustic pressure values as their $p_{r.3}$ (derating factor of 0.3 dB/cm-MHz) because this is the calculation by which diag-

nostic ultrasound devices are regulated and calibrated. Additional studies are necessary to address the issue of acoustic pressure levels at the surface of the lung and species differences in lung hemorrhage that might be related to attenuation caused by the chest wall.

Mean alveolar diameters of approximately 44, 86, 100, and 208 μm have been reported for mice, rabbits, pigs, and human beings, respectively. The sensitivity of lung to ultrasound-induced hemorrhage (mice > rabbits > pigs) determined in earlier studies (W. D.
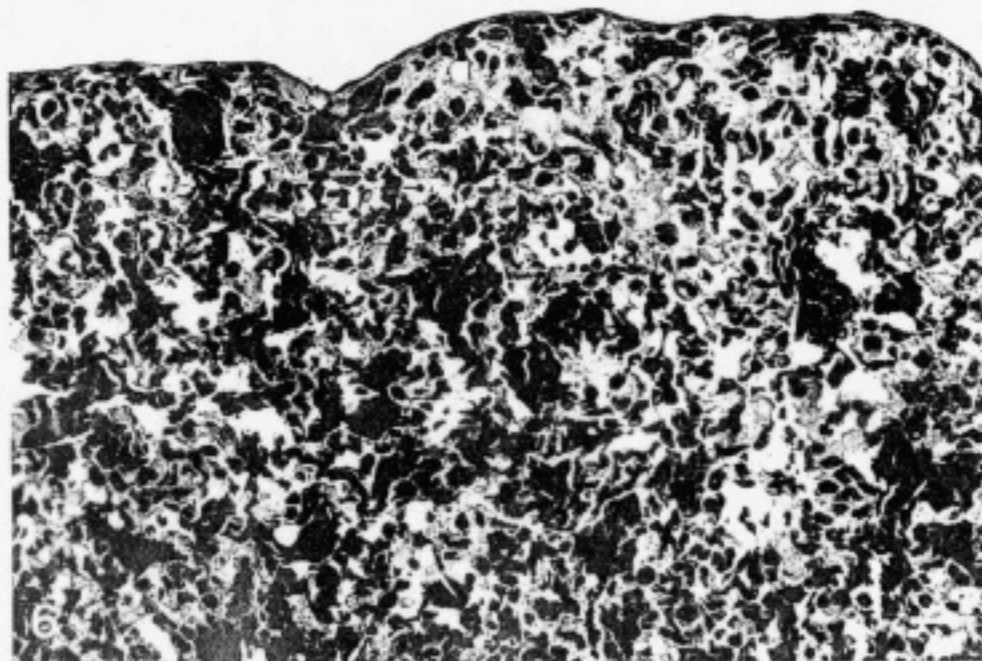

Fig. 6. Lung; mouse. Lung was exposed to pulsed-wave ultrasound at 2.9 MPa for 5 minutes. Alveolar hemorrhage consisted of abundant cells admixed with scant quantities of plasma. Similar lesions were observed in rabbit lung; lesions were not produced in pig lung following exposure to pulsed-wave ultrasound. HE. Bar = 20 μm.

O'Brien, Jr. and J. F. Zachary, in preparation)[12,13] parallels these anatomic differences. Lung compliance or the ability of the lung to stretch and return to normal size following overinflation and its relationship to pleural thickness also may play a role in a species sensitivity to ultrasound-induced lung hemorrhage.[22,23] Rabbit lung is approximately 175 times more compliant than mouse lung; human lung is approximately 4,000 times more compliant than mouse lung. Compliance values for pig lung are not available, but this value probably falls between the values for rabbits and human beings. In addition, mice and rabbits have thin visceral pleura; pigs and human beings have thick visceral pleura.[22] Pleural thickness and possibly collagen, reticulin, and elastin components may reflect an innate defense mechanism against collective overdistention of large numbers of alveoli and tearing of alveolar septa, if bubble formation and expansion (rarefaction/rectified diffusion) are important mechanisms in inducing lung hemorrhage.

Cavitation, a mechanical process affecting gaseous bodies (air bubbles), describes the rapid collapse and expansion of gaseous bodies (<10 μm in diameter) associated with time-varying wave form interaction with the bubble.[3,21] This process results in vitro in the eventual destruction of the bubble and release of gas from the bubble (with possible jet formation) at an extremely high temperature and acoustic pressure. In addition, cavitation also results in enlargement of existing bubbles through fusion with smaller bubbles (rarefaction) and growth (rectified diffusion). The effects of cavitation phenomena have yet to be proven in vivo, but theoretically, rarefaction, rectified diffusion, and/or jet formation could result in mechanical injury (tearing or laceration) or coagulative necrosis of alveolar epithelial cells, connective tissue, and endothelial cells, resulting in lung hemorrhage.[21] A related light and electron microscopic study of pulsed-wave ultrasound in mice suggested that hemorrhage was caused by lysis of both epithelial and endothelial cells.[14] Ultrastructural analysis of lung tissue from our studies is in progress.

Lung hemorrhage induced by continuous- and pulsed-wave ultrasound occurred in the path of the sound beam; thus, lobe specificity for hemorrhage in all species was related to the anatomic positioning of the animal in relationship to the transducer. Target lung lobes (edges of lobes) that had hemorrhage were the areas of lung closest to the ultrasound beam and were located directly under the sternum and ribs (continuous-wave ultrasound) or under the intercostal space (pulsed-wave ultrasound). In lung lobes of a few rabbits, the presence of hemorrhage associated with pleural surfaces not in contiguous alignment with the trans-

ducer was difficult to explain. This finding could be the result of direct or reflective spread of the sound wave from the initial focus on the visceral pleura to other sites within the thoracic cavity. A similar phenomenon has been reported in monkeys.[19]

Each focus of hemorrhage, independent of its size, appeared directly related to a pleural surface, suggesting the mechanism of injury resulting in hemorrhage is initiated in or at the pleural surface and then spreads into lung parenchyma. This finding is important because, in theory, sound waves do not pass through lung tissue because of its low density and low sound speed (air filled). To produce hemorrhage within lung, there must be a mechanism to propagate and spread sound waves through lung. The initial focus of hemorrhage probably is in the pleura and contiguous alveoli. Septal damage and resultant hemorrhage into alveoli displaces air and fills alveoli with plasma and cells, an ideal medium for sound waves to spread and induce lesions in unaffected alveolar septa. A recent study that described the timing of exposures in ultrasound-induced hemorrhage in mouse lung supports this hypothesis.[17] This mechanism may propagate lesions more easily in species with anatomic and physiologic properties of lung such as those that exist in mice; however, this mechanism may be limited or inhibited in species such as pigs with anatomic and physiologic properties of lung that can adapt to mechanical or biological injury. Even though sound (acoustic pressure) cannot readily pass through air-filled alveoli, air-filled alveoli are required for the induction of lung hemorrhage by ultrasound. Hemorrhage is not produced in fetal lung exposed to ultrasound in utero, but it is produced by ultrasound in neonatal aerated lung.[9,10]

The differences in quantity of plasma in relationship to numbers of cells and the protein content (degree of eosin staining) of plasma released into alveoli following exposure to the two different wave forms (continuous-versus pulsed-wave) is difficult to explain mechanistically. The presence of large quantities of protein-rich plasma in alveoli suggests that injury induced by continuous-wave ultrasound may have a vasodilatory and/or vasolytic basis. If ultrasound, early in the exposure period, causes increased circulation through and vasodilation of the lung microvasculature, then lytic lesions affecting alveolar septa could explain the protein-rich plasma in alveoli. In addition, ultrasound could cause vascular leakage associated with alteration of the permeability of endothelial cell tight junctions, resulting initially in leakage of plasma into alveoli followed later by lysis of alveolar septa and hemorrhage.

In summary, both continuous- and pulsed-wave ultrasound produce hemorrhage with similar macroscopic and microscopic features in mice, rabbits, and

pigs; however, there is a difference in the microscopic character of hemorrhage (ratio of plasma to cells) induced by the two wave forms. These findings suggest that physical/biological injury to the microvasculature may be a common mechanism for ultrasound-induced hemorrhage in lung. The dissimilarity in the character of the hemorrhage may be a reflection of differences in the interactions of acoustic pressure from the two different wave forms with endothelial tight junctions or cell membranes. The sequence of events associated with this potential mechanism appears related to initial damage at the visceral pleura and contiguous alveoli, resulting in injury or necrosis and subsequent alveolar hemorrhage. The sound beam then travels through fluid-filled areas (hemorrhage) and reaches normal air-filled alveoli, resulting in injury or necrosis to alveolar septa and subsequent alveolar hemorrhage. This mechanism appears to be propagated in lung by blood-filled alveoli. Injury may be mediated through direct physical (mechanical) damage as a direct result of energy in the sound wave or by more complicated mechanisms related to cavitation-like activity at the cellular level. The overall sensitivity of a species to ultrasound may be determined by anatomic and/or physiologic properties such as alveolar diameter, thickness of alveolar septa, lung compliance, or pleural thickness or by a combination of these factors.

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