Superthreshold Behavior and Threshold Estimation of Ultrasound-Induced Lung Hemorrhage in Pigs: Role of Age Dependency

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***Abstract*—Age-dependent threshold and superthreshold behavior of ultrasound-induced lung hemorrhage were investigated with 116 2.1±0.3-kg neonate crossbred pigs (4.9±1.6 days old), 103 10±1.1-kg crossbred pigs (39±5 days old), and 104 20±1.2-kg crossbred pigs (58±5 days old). Exposure conditions were: 3.1 MHz, 10-s exposure duration, 1-kHz pulse repetition frequency (PRF), and 1.2-μs pulse duration. The in situ (at the pleural surface) peak rarefactional pressure ranged between 2.2 and 10.4 MPa with either eight or nine acoustic pressure groups for each of the three pig ages (12 pigs/exposure group) plus sham exposed pigs. There were no lesions in the shams. Pigs were exposed bilaterally with the order of exposure (left then right lung, or right then left lung) and acoustic pressure both randomized. Pig age was not randomized. Individuals involved in animal handling, exposure, and lesion scoring were blinded to the exposure condition. Logistic regression analysis was used to examine the dependence of the lesion incidence rates on in situ peak rarefactional pressure, left versus right lung exposure, order of exposure (first versus second), and age in three age groups. Likewise, lesion depth and lesion root surface area were analyzed using Gaussian tobit regression analysis. A significant threshold effect on lesion occurrence was observed as a function of age; younger pigs were less susceptible to lung damage given equivalent in situ exposure. Overall, the oldest pigs had a significantly lower threshold (2.87±0.29 MPa) than middle-aged pigs (5.83±0.52 MPa). The oldest pigs also had a lower threshold than neonate pigs (3.60±0.44 MPa). Also, an unexpected result was observed. The ultrasound exposures were bilateral, and the threshold results reported above were based on the lung that was first exposed. After the first lung was exposed, the pig was turned over and the other lung was exposed to the same acoustic pressure. There was a significant decrease (greater than the confidence limits) in occurrence thresholds: 3.60 to 2.68, 5.83 to 2.97, and 2.87 to 1.16 MPa for neonates, middle-aged, and oldest pigs, respectively, in the second lung exposed. Thus, a subtle effect in lung physiology resulted in a major effect on lesion thresholds.**

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I. Introduction

Clinical diagnostic ultrasound is one of the most widely used and safest imaging modalities available in medical practice. Concerns for its safety recently were raised and addressed by members of the bioeffects research community [1]. These concerns are the result of a number of ultrasound-induced lung hemorrhage studies in mice, rats, rabbits, monkeys, and pigs [1]–[22] at exposure conditions similar to those used for scanning in humans. Two categories of study have been used to provide insight into extrapolating the laboratory findings to humans. One of the categories investigated the physical mechanisms responsible for ultrasound-induced lung hemorrhage including physical variables such as acoustic pressure [2], [5], [6], [8]–[15], [17], [19], [21], [22], center frequency [2], [9], [15], [19], [21], pulse duration [2], pulse repetition frequency [2], [5], [6], [18], exposure duration [3], [5], [6], [12], [14], [18], beam shape and/or size [2], [21], temperature [13] and hydrostatic pressure [17], and biophysical endpoints such as heating [3], [23] and cavitation [3], [11], [17], [24], [25]. The other category has investigated the biological mechanisms responsible for ultrasound-induced lung hemorrhage, including biological variables such as species [9], [15], [18], [19], species age [8], [13], [14], lesion size and/or histological observations [4], [5], [8], [9], [10], [13], [14], [20], cardiopulmonary function [22] and lung inflation [26].

The study reported herein evaluates one of the biological mechanisms responsible for ultrasound-induced lung hemorrhage, that is, age dependency in pigs for which there has been several conflicting observations. Monkey studies [8] suggested that animals ranging in age from 3 months to 5 years (mean age of 2.5 yrs) had a greater propensity for the occurrence of multiple well-demarcated circular hemorrhagic foci (0.1–1.0 cm) from exposure to a clinical scanner under maximum output, as compared to older animals (up to 16 years). Mouse studies [13] were reported to have an age-independent threshold for animals ranging in age from 24 hours to 10 weeks but, at superthreshold exposure levels (levels that exceed threshold conditions to produce ultrasound-induced lung hemorrhage), a greater extent of damage in older compared to the younger mice was observed. For the three age groups (24- to 36-hour-old, 14-day-old, 8- to 10-week-old), the in situ (at the pleural surface) peak compressional pressure occurrence threshold estimates were 0.63, 0.92, and

0.72 MPa, respectively, and were reported not to be significantly different. However, the in situ peak compressional pressure surface area threshold estimates were 1.35, 1.24, and 1.05 MPa, respectively, and were reported to be marginally significant. The authors' overall conclusion was that the lung hemorrhage thresholds were the same within the reliability of the data. However, at superthreshold exposure conditions, the lesion's occurrence and surface area were the smallest for the 24- to 36-hour-old mice, and largest for the 8- to 10-week-old mice at the same acoustic pressure, suggesting an age dependency.

Two crossbred pig studies by the same research group using neonate (1-day-old) [10] and young (10-day-old) [14] pigs, and using the same experimental procedures, reported an age-independent threshold. An estimate of the in situ peak rarefactional pressure threshold was 0.9 MPa at 2.3 MHz. However, it was not possible to produce lung damage in 10- to 12-week-old crossbred pigs at estimated in situ peak rarefactional pressures of 1.8 MPa at 3 MHz and 2.2 MPa at 6 MHz [15]. This difference suggested an age-dependent effect.

Thus, a principle motivation for this study was the lack of consistency observed relative to ultrasound-induced lung hemorrhage as a function of the animal's age. Monkey studies suggested that young monkeys were more sensitive than old [8]. Mouse studies suggested an age-independent threshold, and an age-dependent sensitivity under superthreshold exposure conditions with young mice more sensitive than old [13]. Pig studies suggested an age-independent threshold [10], [14]; but, when compared to another study [15], suggested young pigs were more sensitive than old. If the young pigs were in fact more sensitive than old to ultrasound-induced lung hemorrhage, then a potentially significant medical risk might be present for neonate humans. However, if thresholds were indeed age independent, then the extrapolation to humans might be placed on a more firm scientific basis because the mechanism that initiates lung damage might have a common cause. In either case, a more thorough evaluation of ultrasound-induced lung hemorrhage as a function of age is called for. Therefore, the study reported herein estimated the in situ peak rarefactional pressure threshold levels and the sensitivity to lung damage at superthreshold exposure conditions in crossbred pigs at three ages, 4.9±1.6-, 39±5-, and 58±5-day-old.

II. Materials and Methods

A. Exposimetry

Ultrasonic exposures were conducted using a focused, 51-mm-diameter, lithium niobate ultrasonic transducer (Valpey Fisher, Hopkinton, MA). Water-based (degassed water, 22°C) pulse-echo ultrasonic field distribution measurements were performed according to established procedures [19], [27] (Table I).

TABLE I
Water-Based Pulse-Echo Ultrasonic Field Distribution Results for the 51-mm-diameter f/1 Focused Lithium Niobate Ultrasonic Transducer.

Pulse-echo quantity	Value
Center frequency (MHz)	3.1
Fractional bandwidth (%)	15
Focal length (mm)	56
−6-dB focal beamwidth (μm)	610
−6-dB depth of focus (mm)	5.9

An automated procedure, based on established standards [28], [29], was used to routinely calibrate the ultrasound fields [19], [30], [31]. Briefly, the source transducer's drive voltage was supplied by a RAM5000 (Ritec, Inc., Warwick, RI). A calibrated PVDF membrane hydrophone (Marconi Model Y-34-6543, Chelmsford, UK) was mounted to the computer-controlled micropositioning system (Daedal, Inc., Harrisburg, PA). The hydrophone's signal was digitized with an oscilloscope (500 Ms/s, LeCroy Model 9354TM, Chestnut Ridge, NY), the output of which was fed to the same computer (Dell Pentium II, Dell Corporation, Round Rock, TX) that controlled the positioning system. Off-line processing (Matlab, The Mathworks, Natick, MA) yielded the peak water-based rarefactional pressure $p_{r(\text{in vitro})}$ and the peak water-based compressional pressure $p_{c(\text{in vitro})}$ (Table II). The mechanical index (MI) also was determined from the measurement procedure [28]; the MI = $p_{r.3}/\sqrt{f_c}$ where $p_{r.3}$ is the derated (0.3 dB/cm-MHz) peak rarefactional pressure [in megapascal (MPa)] and f_c is the center frequency (in megahertz). The MI is reported because it is a regulated quantity [32], [33] of diagnostic ultrasound systems, and its value is available to system operators. Thus, there is value to provide the MI for each of the exposure settings in order to give general guidance to manufacturers and operators as to the levels we are using in this study. Furthermore, as seen from the MI definition, it is a quantity that must be determined from the water-based measurement process, and cannot be determined from $p_{r(\text{in vivo})}$.

Independent calibrations were performed weekly on the 3.1-MHz focused transducer during the time period of the experiment. One set of calibrations was performed before exposures were initiated each week and one set of calibrations was performed after exposures were concluded for each week. Relative standard deviations $\left(\frac{\text{standard deviation}}{\text{mean}}\right)$ of $p_{r(\text{in vitro})}$ and $p_{c(\text{in vitro})}$ were 6% and 10% (n = 17), respectively. The pulse duration also was measured [29] at every calibration and its mean value (relative standard deviation) was 1.2 (5%) μs.

The in situ (at the pleural surface) peak rarefactional and compressional pressures were estimated for each pig from:

$$p_{r(\text{in situ})} = p_{r(\text{in vitro})} e^{-(A \cdot x)}, \quad (1)$$

TABLE II

MEAN (SEM) VALUES OF THE IN SITU (AT THE PLEURAL SURFACE) PEAK RAREFACTIONAL PRESSURE $p_{r(\text{in situ})}$ AND IN SITU PEAK COMPRESSIONAL PRESSURE $p_{c(\text{in situ})}$ AND THEIR RESPECTIVE LESION OCCURRENCE AND MEAN (SEM) VALUES OF THE LESION DEPTH AND SURFACE AREA.*

Number of animals	$p_{r(\text{in situ})}$ (SEM) (MPa)	$p_{c(\text{in situ})}$ (SEM) (MPa)	Mechanical index	% Lesion occurrence (uncertainty)	Mean (SEM) lesion depth (mm)	Mean (SEM) lesion area (mm^2)
			5-day pigs			
8 (sham)	0.25 (0.01)	0.32 (0.01)	0.12	0	—	—
12	2.6 (0.03)	3.4 (0.04)	1.3	4 (6)	0.01 (0.01)	0.01 (0.01)
12	3.4 (0.07)	5.1 (0.10)	1.7	8 (8)	0.18 (0.14)	1.16 (1.14)
12	4.5 (0.08)	7.8 (0.14)	2.1	8 (8)	0.05 (0.03)	0.10 (0.07)
12	5.6 (0.06)	11.0 (0.12)	2.5	42 (14)	0.31 (0.08)	0.51 (0.20)
12	6.4 (0.12)	15.1 (0.27)	2.9	45 (14)	0.45 (0.11)	1.68 (0.50)
12	7.8 (0.10)	19.9 (0.27)	3.3	79 (12)	0.71 (0.11)	1.89 (0.31)
12	8.4 (0.13)	22.2 (0.35)	3.7	95 (6)	1.10 (0.10)	4.95 (0.42)
12	9.6 (0.16)	25.3 (0.42)	4.1	95 (6)	1.57 (0.23)	4.96 (0.71)
12	10.4 (0.12)	27.2 (0.31)	4.5	95 (6)	1.34 (0.15)	4.94 (0.42)
			39-day pigs			
6 (sham)	0.17 (0.01)	0.21 (0.01)	0.12	0	—	—
12	3.9 (0.11)	7.8 (0.22)	2.5	0	—	—
12	4.9 (0.07)	12.0 (0.16)	3.0	8 (8)	0.05 (0.04)	0.26 (0.18)
12	5.7 (0.11)	15.1 (0.29)	3.5	25 (13)	0.32 (0.13)	1.17 (0.50)
12	6.5 (0.10)	17.3 (0.26)	4.1	33 (14)	0.43 (0.15)	1.50 (0.60)
12	7.6 (0.16)	19.8 (0.41)	4.6	42 (14)	0.59 (0.16)	3.38 (1.00)
13	8.3 (0.27)	20.9 (0.69)	5.1	65 (14)	1.41 (0.25)	6.38 (1.18)
12	8.4 (0.22)	20.3 (0.54)	5.7	83 (11)	2.03 (0.27)	8.67 (1.08)
12	9.5 (0.14)	22.2 (0.32)	6.2	92 (8)	2.77 (0.39)	11.8 (1.48)
			58-day pigs			
8 (sham)	0.09 (0.002)	0.12 (0.003)	0.12	0	—	—
12	2.2 (0.07)	4.3 (0.14)	2.5	0	—	—
12	2.6 (0.07)	6.3 (0.17)	3.0	17 (11)	0.23 (0.13)	1.52 (0.80)
12	3.2 (0.08)	8.4 (0.21)	3.5	38 (14)	0.46 (0.14)	2.27 (0.85)
12	3.7 (0.09)	9.8 (0.24)	4.1	50 (14)	0.77 (0.18)	6.69 (2.70)
12	4.2 (0.09)	10.9 (0.24)	4.6	54 (14)	1.03 (0.24)	6.61 (1.74)
12	4.5 (0.11)	11.3 (0.28)	5.1	54 (14)	1.05 (0.24)	9.21 (2.27)
12	4.5 (0.10)	11.0 (0.24)	5.7	67 (14)	1.34 (0.28)	7.44 (1.81)
12	5.2 (0.16)	12.1 (0.38)	6.2	96 (6)	2.76 (0.34)	16.5 (2.38)

*All pigs were exposed to pulsed ultrasound (1-kHz pulse repetition frequency, 10-s exposure duration, 1.2-μs pulse duration). The sham exposure conditions used a pulse repetition frequency of 10 Hz. Mean values of the MI, as measured according to the applicable standard [28], are provided because the MI is a regulated quantity of diagnostic ultrasound equipment.

and

$$p_{c(\text{in situ})} = p_{c(\text{in vitro})} e^{-(A \cdot x)}, \tag{2}$$

respectively, where $p_{r(\text{in vitro})}$ and $p_{c(\text{in vitro})}$ are the measured maximum water-based peak rarefactional and compressional pressures. The mean attenuation coefficient, A, of the pig's intercostal tissue used herein was determined in an independent study from 15 5.3±2.3-day-old (weight: 2.2±0.4 kg), 19 31±6-day-old (weight: 8.5±0.5 kg), and 15 61±3-day-old (weight: 20±1.1 kg) crossbred pigs [34]. In that study, the attenuation coefficient of intercostal tissue was shown to be independent of the pig age at 3.1 MHz (2.02 dB/cm-MHz, SEM = 0.082, n = 49). However, the frequency-dependent regression analyses were significantly different as a function of pig age. Therefore, the attenuation coefficients used to estimate $p_{r(\text{in situ})}$ and $p_{c(\text{in situ})}$ (1) and (2) were different for the three pig age groups, e.g., 6.85, 5.61, and 6.48 dB/cm (at 3.1 MHz) for the 5-day-old, 39-day-old, and 58-day-old pigs, respectively [34]. Also, each pig was ultrasonically exposed bilaterally. The measured individual intercostal tissue thicknesses, x, were used in (1) and (2) to calculate $p_{r(\text{in situ})}$ and $p_{c(\text{in situ})}$. And, even though the intercostal tissue attenuation coefficients were measured at 22°C and the in vivo intercostal tissue is approximately 37°C, a separate temperature-dependent attenuation coefficient study showed that the intercostal tissue attenuation coefficient of the pig is independent of temperature between 22 and 37°C [35].

B. Animals

The experimental protocol was approved by the campus' Laboratory Animal Care Advisory Committee and satisfied all campus and National Institutes of Health rules

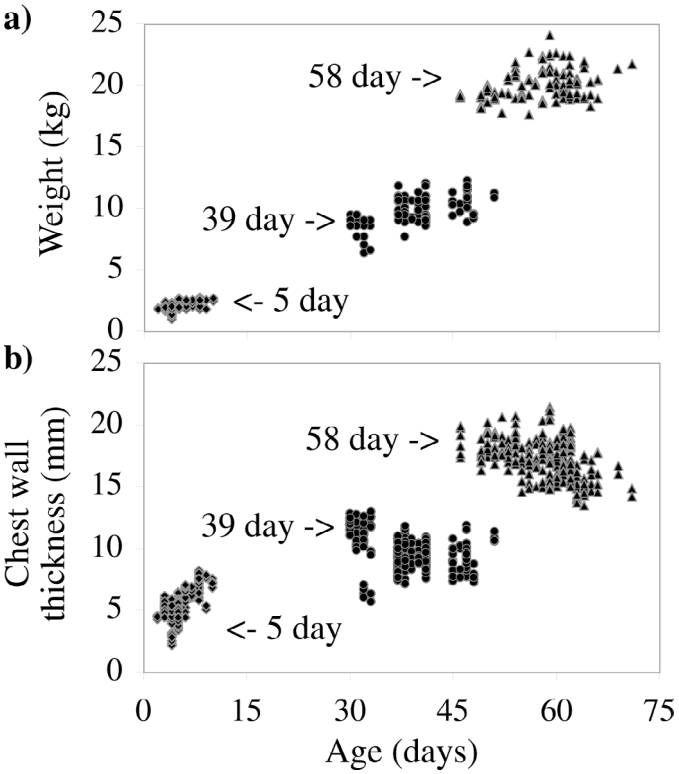

Fig. 1. Weight (a) and chest wall thickness (b) as a function of the 323 crossbred pig's age evaluated in this study.

for the humane use of laboratory animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care, Rockville, MD (AAALAC)-approved animal facility, placed in groups of one to five in raised deck pens with expanded metal floors, and provided food and water ad libitum. The AAALAC is a private nonprofit organization that promotes the humane treatment of animals in science through a voluntary accreditation program.

There were 116 4.9±1.6-day-old (mean±SD) neonate crossbred pigs (weight: 2.1±0.3 kg, chest wall thickness: 5.3±1.1 mm); 103 39±5-day-old crossbred pigs (weight: 9.8±1.1 kg, chest wall thickness: 9.7±1.4 mm), and 104 58±5-day-old crossbred pigs (weight: 20±1.2 kg, chest wall thickness: 17±1.7 mm) (Fig. 1). Animal weights were measured at the time of the experiment, and animal ages were determined from exact birth date. The selection of these age (weight) groups was somewhat arbitrary because the mechanisms by which ultrasound produces lung hemorrhage are not understood, and thus the selection could not have a specific biological basis. Nevertheless, there are maturation changes as the animal ages, particularly those based on recognized pig production time points. These time points are linked to biological events, such as disease susceptibly and physical and sexual maturation that occur within defined production time points. Such production categories include preweaning pigs (our 5-day-old group), postweaning pigs (our 39-day-old group), feeder pigs (our 58-day-old group). There is also a practical upper limit because pigs that weigh more than 70 pounds become difficult to work with and can pose a risk to scientific staff. These categories can be used to separate pigs into age groups in experimental studies. The thickness of the visceral pleura, and to a lesser extent alveolar septa, increase with age in all mammalian species as the result of developmental maturation [36]–[39]. Maturation results from the synthesis and deposition of structural proteins to form organized bundles of fibrous connective tissue and elastic fibers in these areas. Differences in the thickness of visceral pleura and alveolar septa also are determined by the size of the species. Mammalian species demonstrate an allometric relationship based on body weight; thus, larger animals (pigs) will have more structural fibers in corresponding areas than smaller animals (mice). The thickness of the visceral pleura and alveolar septa in adult pigs closely parallel the same structures in humans.

All pigs were obtained from the University of Illinois Veterinary Research Farm (Urbana, IL). These three age groups will be referred to as 5-day, 39-day, and 58-day pigs for convenience. In each age group, pigs were assigned randomly to eight (39-day and 58-day) or nine (5-day) acoustic pressure levels (Table II), each with 12 pigs per pressure level (except one of the 39-day pig groups that had 13 pigs per pressure level). Of the total 323 pigs, eight 5-day pigs, six 39-day pigs, and eight 58-day pigs were shams, and they were included in the randomized design. No lesions were produced in the exposed sham pigs. The individuals involved in pig handling, exposure, necropsy, and lesion scoring were blinded to the exposure condition. The exposure condition for each pig was revealed only after the final results were tabulated.

Pigs were anesthetized with an intramuscular injection of ketamine hydrochloride (2.2 mg/kg), xylazine (2.2 mg/kg), and tiletamine/zolazepam (4.4 mg/kg). The skin of the thorax (both sides) was shaved with an electric clipper, followed by a depilatory agent (Nair® Carter-Wallace, Inc., New York, NY) to maximize sound transmission. A black dot was placed on the skin over the ribs at approximately the fifth to sixth rib on both sides to guide the lateral positioning of the ultrasonic beam. Anesthetized pigs were placed in right or left lateral recumbancy and a stand-off tank positioned in contact with the skin (Fig. 2). Mineral oil was used as the coupling agent on the skin surface. All pigs were exposed bilaterally. The ultrasound transducer, placed in the stand-off tank that contained degassed water at 30°C, was aligned with the black dot prior to each exposure. The low-power pulse-echo capability of the exposure system (RAM5000) displayed on an oscilloscope was used to align the axial center of the focal region to within 1 mm of the lung surface (see sham in Table II for these low-level ultrasonic pressure levels). Also, the pulse repetition frequency was reduced to 10 Hz during this alignment procedure. Thus, the ultrasonic beam was approximately perpendicular to the skin at the position of the black dot, with the beam's focal region at the lateral surface of the lung, and approximately normal to the lung's pleural surface. After either the left or right lung was exposed, the pig was turned over, the transducer was re-

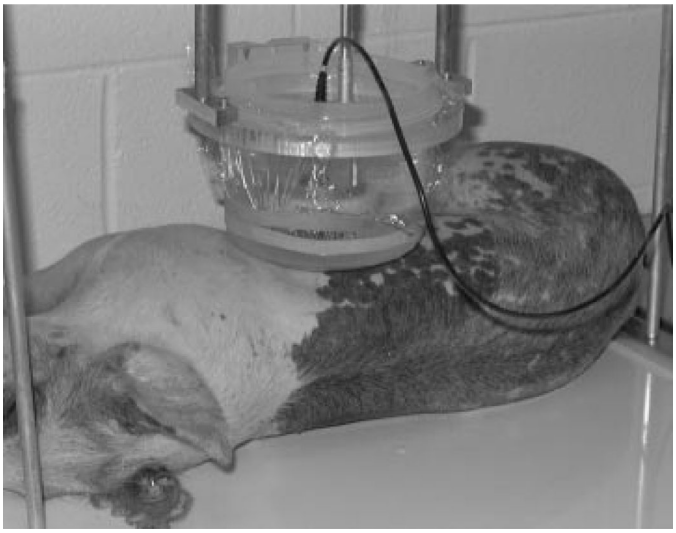

Fig. 2. Picture of a pig with the stand-off tank.

aligned again as described above, and the pig's other lung was exposed. The acoustic pressure level was the same for both the left and right lungs of each pig. The order (left then right lung, or right then left lung) was randomized with an equal number of exposures for each order.

The distance between the transducer and the lung surface did not vary by more than 1–2 mm because the water-filled stand-off tank was placed gently on the pig's lateral aspect, thus limiting chest expansion in that direction. The −6-dB depth of focus is 5.9 mm so the lung surface remained within the focal region as a function of the animal's breathing. Also, the orientation of the chest wall surface in contact with the water-filled stand-off tank did not visually change as a function of breathing. Because the chest wall surface and the lung surface track each other, the orientation between the beam axis and the pleural surface did not change. In addition, the center-to-center rib spacing of pigs ranged between about 8 mm for a 5-day pig to 17 mm for a 61-day pig [34]; with a beamwidth at the plueral surface of 610 μm, there was adequate space between the ribs for the sound field. Therefore, the observations and findings reported herein are not believed to be a function of lung surface or rib orientation, position, or movement relative to the ultrasonic field. That is, animal motion during breathing was neither a factor during the alignment procedure nor the actual exposure procedure.

Each lung was exposed to 3.1-MHz pulsed ultrasound with a pulse repetition frequency of 1 kHz, an exposure duration of 10 s and a pulse duration of 1.2 μs. The 10-s exposure duration was used to simulate incidental exposure to lung tissue because, in clinical practice, the lung is generally not intentionally exposed to diagnostic ultrasound. Following exposure, pigs were euthanized under anesthesia with an intravenous injection of sodium pentobarbital (0.22 mL/kg).

A midline incision was made to open the chest cavity. The left and right thoracic walls were opened, and the thicknesses of the intercostal tissue (skin, fat, fascia, muscle, and parietal pleura) were measured on both sides with a digital micrometer (accuracy: 10 μm; Mitutoyo Corp., Kawasaki, Kanagawa, Japan) at the locations of exposure. These chest wall measurements were used for later calculation of the in situ ultrasonic pressures at the visceral pleural surface (1) and (2).

The lungs were removed from each pig, rinsed in 0.9% sodium chloride, examined grossly for the presence or absence of a lesion, then photographed digitally. The base of the lesion originated at the visceral pleural surface and was elliptical in shape. The lesion extended into lung parenchyma to form its apex at varied depths within the lung. A section of the lung where the lesion appeared, or the target area where the lesion would have appeared, was trimmed away from the lung; placed in a sterile, 50 mL, clear polypropylene centrifuge tube; then fixed by immersion in 10% neutral-buffered formalin for a minimum of 24 hours. After total fixation, the elliptical dimensions of lung lesions at the visceral pleural surface were measured with a digital micrometer (accuracy: 10 μm), where "a" was the length of the semimajor axis and "b" was the length of the semiminor axis. The lesions then were bisected and the depth "d" of the lesion within the lung was measured. In animals in which the depth of the lesion was not visually discernible, the depth was determined from measurements made on histologic sections with a slide micrometer. The surface area (πab) and volume (πabd/3) of the lesion were calculated for each lung. Each half of the bisected lesion was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and evaluated microscopically.

C. Statistics

Logistic regression analysis was used to examine the dependence of the lesion incidence (occurrence) rates on in situ peak rarefactional pressure, $p_{r(\text{in situ})}$, left versus right lung, order of exposure (first versus second) and age in three categories (5-day, 39-day and 58-day). In this analysis, the log-odds of an event (i.e., occurrence of a lesion) is modeled as a linear function of the variables in the study; and, equivalently, the probability of an event is modeled as a logistic transformation of a linear model [40]. Let P denote the probability of a lesion. Then the logistic regression model has the form:

$$\mathrm{P} = \mathrm{H}\{(\mathrm{Coef}_1)(\mathrm{Var}_1) + (\mathrm{Coef}_2)(\mathrm{Var}_2) + \cdots + (\mathrm{Coef}_\mathrm{k})(\mathrm{Var}_\mathrm{k})\}, \quad (3)$$

where $\mathrm{H}\{\mathrm{x}\} = (1 + e^{-x})^{-1}$, $\mathrm{Var_i}$ denotes a variable in the model, and $\mathrm{Coef_i}$ denotes the corresponding coefficient. Maximum likelihood estimates of the coefficients (Table III) were obtained using the data on presence or absence of lesions irrespective of lesion size.

The logistic regression computations were performed using the S-Plus® (Insightful Corp., Seattle, WA) function glm. Variables were selected by initially including all experimental variables and pair-wise interactions in the

TABLE III
SUMMARY OF LOGISTIC REGRESSION MODEL FOR OCCURRENCE OF LESIONS IN 301 CROSSBRED PIGS.

Variable	Coefficient	Standard error	z value
Intercept	−7.23	1.06	−6.83
$p_{r(\text{in situ})}$	1.19	0.166	7.17
Order	2.18	1.09	1.99
AGE1	−1.52	1.28	−1.19
AGE2	−0.339	1.29	−0.26
$p_{r(\text{in situ})}$* order	−0.400	0.163	−2.45
$p_{r(\text{in situ})}$* AGE1	−0.194	0.161	−1.20
$p_{r(\text{in situ})}$* AGE2	0.423	0.245	1.73
Order*AGE1	1.86	0.663	2.81
Order*AGE2	−1.04	0.652	1.60

model, then using backward selection to eliminate insignificant interactions and main effects. Logistic regression estimates were transformed to yield estimates and confidence intervals for two effective dose (ED) levels, the ED05 and ED50 levels (i.e., the in situ peak rarefactional pressure associated with 5% and 50% probabilities of lesions, respectively) [19], [21], [41]. The ED05 level will be referred to as the ED05 threshold. It is understood that in situ peak rarefactional pressure is a dose in the generalized sense of an exposure quantity rather than in the specific sense of a chemical concentration or radiation dose.

Logistic regression mixed model analysis [42], with a random effect for each crossbred pig, was used to assess whether the two exposure responses (both lungs were exposed to the same $p_{r(\text{in situ})}$ value) for each pig were correlated after adjusting for $p_{r(\text{in situ})}$, age, left versus right lung, and order of exposure (first versus second). The mixed model estimates were computed using the method of Wolfinger and O'Connell [43] as implemented in the SAS macro GLIMMIX (SAS Institute, Inc., Cary, NC).

Depth and root surface area ($\sqrt{\text{surface area}}$) of lesions were analyzed using Gaussian tobit regression using the S-Plus® survReg function. In the tobit regression model, a lung without lesions is recorded as having a lesion of size zero. The model implies that the median lesion size is zero up to a threshold acoustic pressure, and that above the threshold the median size increases linearly [44], [45]. Let Y denote the lesion size (depth or root surface area). Then, according to the tobit regression model, Y is distributed as the non-negative truncation of a Gaussian random variable Z, where Z follows a linear regression model:

$$Z = (\text{Coef}_1)(\text{Var}_1) + (\text{Coef}_2)(\text{Var}_2) + \cdots + (\text{Coef}_k)(\text{Var}_k) + \varepsilon, \quad (4)$$

where ε is Normally distributed with mean zero and variance S^2. Thus $Y = Z$ if $Z > 0$ and $Y = 0$ if $Z \leq 0$. If the right side of (4) is positive, then it is also the conditional median of Y (the lesion size) given $\text{Var}_1, \text{Var}_2, \ldots, \text{Var}_k$ [46].

The tobit model in (4) implies that the probability P of a nonzero observation (here, the probability of a lesion) is given by:

$$P = \Phi\left\{\frac{(\text{Coef}_1)(\text{Var}_1) + (\text{Coef}_2)(\text{Var}_2) + \cdots + (\text{Coef}_k)(\text{Var}_k)}{S}\right\}, \quad (5)$$

where Φ is the standard Normal distribution function, and the coefficients, variables, and error standard deviation S are the same as in (4).

The logistic regression model in (3) uses only presence or absence of lesions, whereas the tobit model in (4) and (5) takes into account the size as well; it uses more information but makes a stronger modeling assumption. Both models yield estimates of threshold probabilities, and comparing results from the two methods provides a check on the tobit model assumptions.

For animals with lesions, the depth or root surface area was included as the response measurement. For animals without lesions, the depth or root surface area was included as a zero value. Graphs of ordered residuals versus normal percentiles indicated the need for a square root transformation of surface area in order to achieve an adequate fit of a linear tobit model. The ED05s and ED50s (in situ peak rarefactional pressure associated with a 5% and 50% incidence rate) were determined by solving (5) for the values of $p_{r(\text{in situ})}$ such that $P = 0.05$ and $P = 0.50$, respectively. Standard errors for these quantities were obtained by first-order Taylor series approximations. Standard errors and confidence intervals were computed in S-Plus® using matrix calculations.

III. RESULTS

A. Results for Chest Wall Thickness

The exposures were bilateral; therefore, chest wall thicknesses were measured on both sides. The mean±SD (range) values of the chest wall thicknesses were: 5.2±1.1 (2.3–8.0) mm (5-day, order 1, n = 116), 5.3±1.1 (2.2-8.2) mm (5-day, order 2, n = 116), 9.7±1.4 (6.3–13.0) mm

(39-day, order 1, n = 103), 9.7±1.5 (5.7–12.8) mm (39-day, order 2, n = 103), 17.3±1.7 (13.7–21.5) mm (58-day, order 1, n = 104), 17.4±1.6 (13.5–20.7) mm (58-day, order 2, n = 104). The designations of order 1 and order 2 refer to the order the lungs were exposed on the same pig. Order 1 denotes the first lung exposed, and order 2 denotes the second lung exposed. An equal number of left and right lungs were exposed for each order.

B. Results for Lesion Occurrence

The mixed model analysis did not detect a significant correlation between incidence (occurrence) of lesions on the two sides after adjusting for $p_{r(\text{in situ})}$, age, and order of exposure (first versus second). Subsequent analyses, therefore, treated the two lung exposures of each animal as independent observations. The logistic regression model for occurrence of lesions [Fig. 3(a)] was highly statistically significant, with a log-likelihood ratio chi-square of 304.4 on 9 degrees of freedom and $p < 0.0001$, indicating that the association between lesions and the variables in the model cannot be explained by chance alone.

Table III gives the coefficient estimate, standard error, and z value $\left(\frac{\text{estimate}}{\text{standard error}}\right)$ for each term in the model. The following variables were statistically significant at level 0.05 in the logistic regression analysis for lesion occurrence:

- in situ peak rarefactional pressure, $p_{r(\text{in situ})}$, in MPa,
- order (order 1 versus order 2),
- interaction between $p_{r(\text{in situ})}$ and age category (AGE0 = "5-day pigs", AGE1 = "39-day pigs", AGE2 = "58-day pigs"),
- interaction between $p_{r(\text{in situ})}$ and order,
- interaction between order and age.

The interactions of age and order with $p_{r(\text{in situ})}$ indicate that the effect of incremental increases in acoustic pressure on the log-odds of a lesion depend on the age of the animal as well as the order of lung exposure. Conversely, the effect of age or order of exposure is dependent on $p_{r(\text{in situ})}$. In assessing the age parameters, it should be noted that AGE0 (5-day pigs) is the reference age category, so the coefficient for AGE1 is the increase in log odds of a lesion for 39-day versus 5-day pigs, and the coefficient for AGE2 (58-day pigs) is the increase in log odds of a lesion for 58-day versus 5-day pigs. The order coefficient is the increment in log odds for order 2 versus order 1 exposure. The coefficient for $p_{r(\text{in situ})}$ is the increase in log odds of a lesion in 5-day pigs (the reference age group) for each 1-MPa increase in $p_{r(\text{in situ})}$. The negative coefficient for $p_{r(\text{in situ})}$*AGE1 suggests that 39-day pigs are less sensitive than 5-day pigs to increases in $p_{r(\text{in situ})}$. The positive coefficient for $p_{r(\text{in situ})}$*AGE2 suggests that 58-day pigs are more sensitive than 5-day pigs to increases in $p_{r(\text{in situ})}$. Similarly, the negative coefficient for $p_{r(\text{in situ})}$*order suggests that order 2 (second lung exposed) is less sensitive than order 1 (first lung exposed) to increases in $p_{r(\text{in situ})}$. The positive coefficient for order*AGE1 suggests that there is a further increase in log

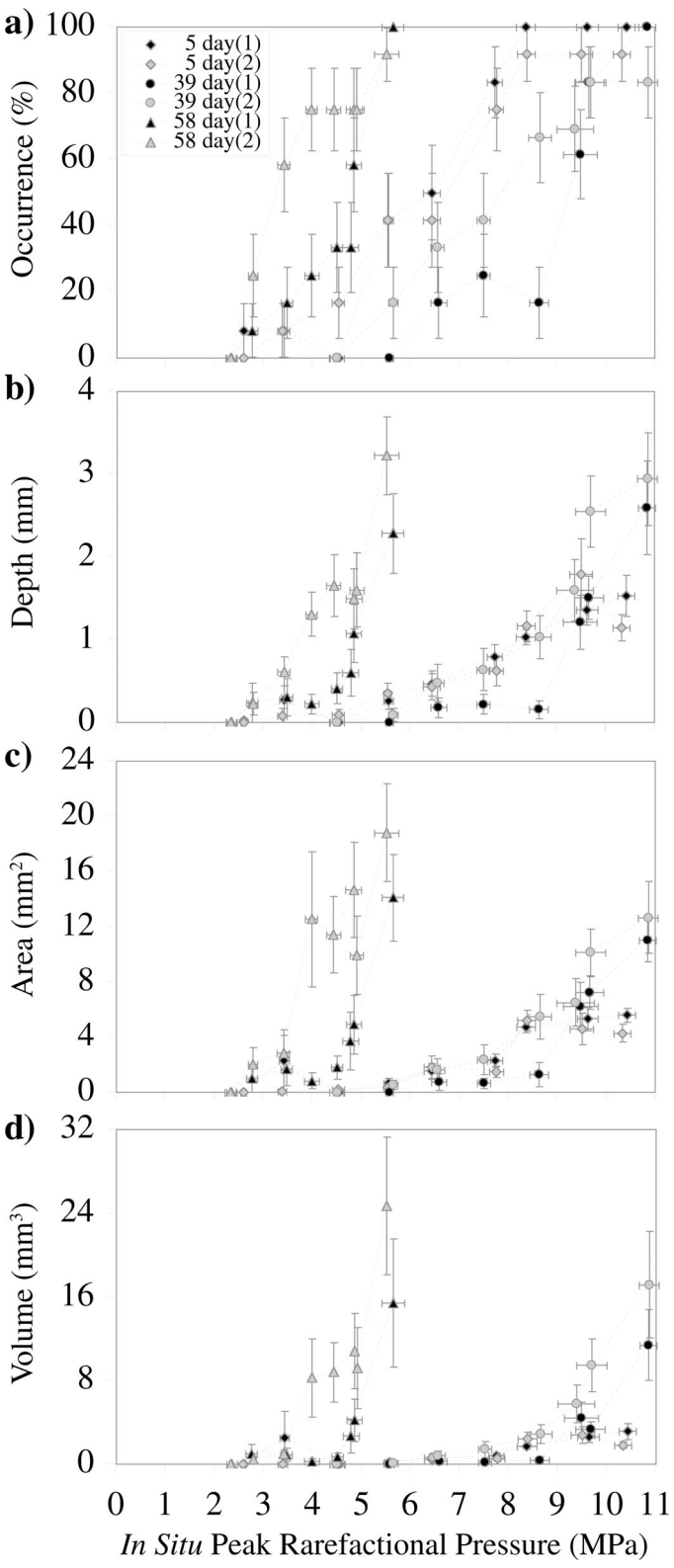

Fig. 3. (a) Lesion occurrence, (b) lesion depth, (c) lesion surface area, and (d) lesion volume as a function of the in situ peak rarefactional pressure. The dashed lines are straight lines connecting the mean values. For each age group (5-day, 39-day, and 58-day pigs), there are two data sets, one represents the first exposed lungs (order 1), denoted by 1, and the other representing the second exposed lungs (order 2), denoted by 2. Error bars are the standard errors of the mean (n = 12).

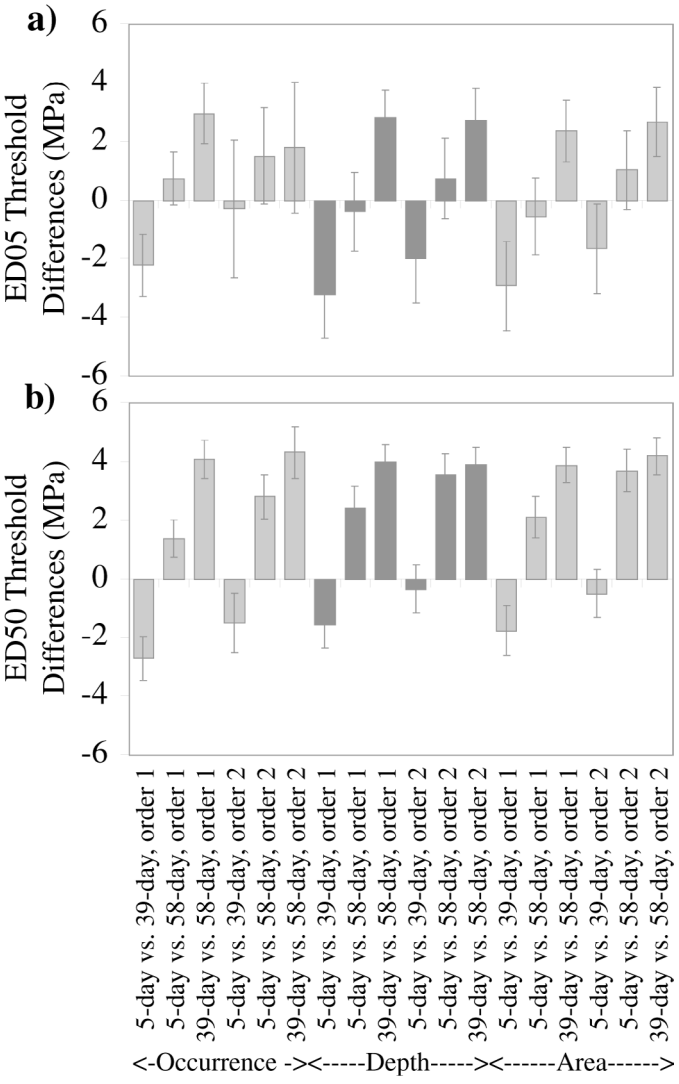

Fig. 4. Paired comparisons of age-specific ED05 threshold (a) and ED50 level (b) differences for occurrence, depth, and surface area of lesions. A positive difference indicates that the first listed pig age has a greater value than the second listed pig age. The confidence bars are the ±1.96 standard errors; a difference is statistically significant if the confidence bar does not cross the zero-difference axis.

odds for order 2 exposure than order 1 exposure for 39-day pigs than that in 5-day pigs. Also, the negative coefficient for order*AGE2 means that there is a further decrease in log odds for order 2 exposure than order 1 exposure in 58-day pigs than that in 5-day pigs.

Table IV gives threshold ED05 and ED50 estimates for occurrence of lesions, broken out by order of exposure and age category. Significant age effects were observed. In particular, for order 1 (first lung exposed), 58-day pigs had significantly lower ED05 thresholds (2.87±0.29 MPa) for lesions in the lung than 39-day pigs (5.83±0.52 MPa). The 58-day pigs also had lower ED05 thresholds than 5-day pigs (3.60±0.44 MPa). The significance of the differences is confirmed in Table V, which provides pairwise differences between age-specific ED05s and ED50s along with the standard errors for the differences. The differences between 5-day pig ED05 thresholds and 58-day pig ED05 thresholds were not statistically significant (Fig. 4). The 39-day pigs had significantly higher ED05 thresholds than the 5-day and 58-day pigs.

C. Results for Lesion Depth

The tobit regression model for lesion depth [Fig. 3(b)] was highly statistically significant, with a log-likelihood ratio chi-square of 331.6 on 8 degrees of freedom and $p < 0.0001$. The same variables were statistically significant at level 0.05 in the lesion depth tobit regression analysis as in the logistic regression analysis of incidence, except that the interaction between $p_{r(\text{in situ})}$ and order was not significant in the tobit analysis. Table VI summarizes the estimated tobit regression model for lesion depth. The log(scale) refers to the natural logarithm of the error standard deviation S of (5). Table IV gives ED05 and ED50 estimates derived from the tobit regression of depth, and Table V provides the estimated pairwise differences and standard errors for the differences between age-specific ED05s and for ED50s. The 58-day pigs had lower estimated ED05s than the 39-day pigs, with order 1 (first lung exposed) estimate of 2.51±0.26 MPa, and order 2 (second lung exposed) estimate of 1.45±0.31 MPa. The differences between 5-day pig ED05 thresholds and 58-day pig ED05 thresholds were not statistically significant. The 39-day pigs had significantly higher ED05 thresholds than the 5-day and 58-day pigs for both orders 1 and 2 (Fig. 4).

D. Results for Lesion Surface Area

The tobit regression model for lesion surface area [Fig. 3(c)] was highly statistically significant, with a log-likelihood ratio chi-square of 337.0 on 8 degrees of freedom and $p < 0.0001$. The same variables were statistically significant at level 0.05 in the root surface area tobit regression analysis as in the logistic regression analysis of incidence, except that the interaction between $p_{r(\text{in situ})}$ and order was not significant in the tobit analysis. Table VII summarizes the estimated tobit regression model for root surface area. The log(scale) refers to the logarithm of the error standard deviation S of (5). Table IV gives ED05 and ED50 threshold estimates derived from the tobit regression of area, and Table V provides the estimated pairwise differences and standard errors for the differences between age-specific ED05s and for ED50s. The 58-day pigs had lower estimated ED05s than 39-day pigs, with order 1 estimate of 2.40±0.25 MPa, and order 2 estimate of 1.15±0.32 MPa. The differences between ED05 thresholds for 5-day and 58-day pigs were not statistically significant, and the 39-day pigs had significantly higher ED05 thresholds than both 5-day and 58-day pigs for both orders 1 and 2 (Fig. 4).

Lesion volume was not evaluated statistically because it is not a direct measure, but it is provided graphically [Fig. 3(d)] for completeness.

E. Results on Weight Dependence

In studying age effects, a possible confounding factor is weight, which is highly correlated with age. Indeed, weight

TABLE IV

ED05 THRESHOLD AND ED50 LEVEL ESTIMATES FOR OCCURRENCE, DEPTH, AND SURFACE AREA OF LESIONS IN 301 CROSSBRED PIGS.

Endpoint	ORDER	Age category	Threshold/level* (MPa)	
			ED05 (Std Err)	ED50 (Std Err)
Occurrence	1	5-day	3.60 (±0.44)	6.08 (±0.27)
	1	39-day	5.83 (±0.52)	8.78 (±0.29)
	1	58-day	2.87 (±0.29)	4.70 (±0.17)
	2	5-day	2.68 (±0.69)	6.40 (±0.33)
	2	39-day	2.97 (±1.06)	7.91 (±0.40)
	2	58-day	1.16 (±0.53)	3.59 (±0.21)
Depth	1	5-day	2.11 (±0.64)	6.95 (±0.34)
	1	39-day	5.32 (±0.41)	8.51 (±0.26)
	1	58-day	2.51 (±0.26)	4.52 (±0.16)
	2	5-day	2.19 (±0.63)	7.02 (±0.34)
	2	39-day	4.19 (±0.45)	7.37 (±0.26)
	2	58-day	1.45 (±0.31)	3.46 (±0.16)
Area	1	5-day	1.84 (±0.62)	6.49 (±0.34)
	1	39-day	4.76 (±0.47)	8.25 (±0.28)
	1	58-day	2.40 (±0.25)	4.37 (±0.16)
	2	5-day	2.17 (±0.60)	6.82 (±0.33)
	2	39-day	3.84 (±0.51)	7.33 (±0.28)
	2	58-day	1.15 (±0.32)	3.12 (±0.18)

*ED05 threshold: obtained by inverting the logistic regression model for the 5% probability or the tobit model for 95% censoring at zero. ED50 level: obtained by inverting the logistic regression model for the 50% probability or the tobit model for 50% censoring at zero.

TABLE V

PAIRED COMPARISONS OF AGE-SPECIFIC ED05 THRESHOLDS AND ED50 LEVELS FOR OCCURRENCE, DEPTH, AND SURFACE AREA OF LESIONS IN 301 CROSSBRED PIGS.*

Endpoint	Order	Comparison	Difference† (MPa)	
			ED05	ED50
Occurrence	1	5-day vs 39-day	−2.22 (±0.55)	−2.71 (±0.39)
	1	5-day vs 58-day	0.74 (±0.47)	1.38 (±0.32)
	1	39-day vs 58-day	2.96 (±0.53)	4.09 (±0.33)
	2	5-day vs 39-day	−0.29 (±1.20)	−1.50 (±0.52)
	2	5-day vs 58-day	1.51 (±0.84)	2.81 (±0.39)
	2	39-day vs 58-day	1.80 (±1.14)	4.32 (±0.45)
Depth	1	5-day vs 39-day	−3.21 (±0.76)	−1.55 (±0.42)
	1	5-day vs 58-day	−0.40 (±0.69)	2.43 (±0.37)
	1	39-day vs 58-day	2.81 (±0.48)	3.99 (±0.30)
	2	5-day vs 39-day	−2.00 (±0.77)	−0.34 (±0.42)
	2	5-day vs 58-day	0.74 (±0.70)	3.56 (±0.37)
	2	39-day vs 58-day	2.73 (±0.55)	3.91 (±0.30)
Area	1	5-day vs 39-day	−2.92 (±0.78)	−1.76 (±0.43)
	1	5-day vs 58-day	−0.56 (±0.67)	2.12 (±0.36)
	1	39-day vs 58-day	2.36 (±0.53)	3.88 (±0.31)
	2	5-day vs 39-day	−1.66 (±0.78)	−0.50 (±0.42)
	2	5-day vs 58-day	1.02 (±0.68)	3.70 (±0.37)
	2	39-day vs 58-day	2.68 (±0.60)	4.20 (±0.32)

*A positive difference indicates that the second listed pig age had a lower value than the first listed pig age.
†ED05 threshold: obtained by inverting the logistic regression model for the 5% probability or the tobit model for 95% censoring at zero. ED50 level: obtained by inverting the logistic regression model for the 50% probability or the tobit model for 50% censoring at zero.

TABLE VI
SUMMARY OF GAUSSIAN TOBIT MODEL FOR DEPTH OF LESIONS IN 301 CROSSBRED PIGS.

Variable	Coefficient	Standard error	z value
Intercept	−3.55	0.443	−8.02
$p_{r(\text{in situ})}$	0.511	0.054	9.51
Order	−0.037	0.241	−0.15
AGE1	−3.05	0.781	−3.91
AGE2	−2.01	0.73	−2.76
$p_{r(\text{in situ})}$* AGE1	0.266	0.087	3.05
$p_{r(\text{in situ})}$* AGE2	0.720	0.134	5.36
Order*AGE1	0.920	0.356	2.58
Order*AGE2	1.34	0.355	3.78
Log(scale)	0.407	0.045	9.15

TABLE VII
SUMMARY OF GAUSSIAN TOBIT MODEL FOR SQUARE ROOT SURFACE AREAS OF LESIONS IN 301 CROSSBRED PIGS.

Variable	Coefficient	Standard error	z value
Intercept	−4.55	0.575	−7.92
$p_{r(\text{in situ})}$	0.702	0.070	10.0
Order	−0.232	0.316	−0.74
AGE1	−3.16	0.99	−3.19
AGE2	−2.68	0.94	−2.86
$p_{r(\text{in situ})}$* AGE1	0.233	0.112	2.08
$p_{r(\text{in situ})}$* AGE2	0.953	0.173	5.50
Order*AGE1	1.098	0.464	2.37
Order*AGE2	2.30	0.464	4.95
Log(scale)	0.684	0.046	15.0

and age often are used interchangeably to describe the level of maturity of a pig. Weight ranges originally classified the three age groups of pigs. The resultant grouping showed some overlap in age between the 39-day and 58-day groups. The age ranges were 2–10 days (5-day group), 30–51 days (39-day group), and 46–71 days (58-day group). To check whether this had a substantial influence on the results, the analysis was repeated after restricting the groups to 2-week intervals of nonoverlapping age categories: 2–10 days (neonate), 33–47 days (reduced 39 day), and 53–67 days (reduced 58 day). The model coefficients and threshold estimates were nearly the same as those obtained from the full data set, so only the full data results were presented.

An additional analysis was run on the 58-day group, including $p_{r(\text{in situ})}$ and weight as variables in the logistic regression model. Although $p_{r(\text{in situ})}$ was significant in the model, weight was not significant.

IV. Discussion

The objective of this study was to assess the role of pig age on the threshold and superthreshold behavior of ultrasound-induced lung hemorrhage because of reports from previous findings [8], [10], [13]–[15] that there was a lack of consistency observed relative to ultrasound-induced lung hemorrhage as a function of the animal's age.

A significant ED05 threshold effect was observed as a function of age and showed that the youngest (5-day) and oldest (58-day) pigs studied are more susceptible to lung damage than 39-day pigs given equivalent $p_{r(\text{in situ})}$ exposure. In general for all three measures (lesion occurrence, depth, and area), the youngest (5-day) pigs and oldest (58-day) pigs had significantly lower ED05 thresholds than 39-day pigs. Also, the ED05 thresholds for the youngest and oldest pigs were not significantly different. The six left bars in Fig. 5 graphically show the pig ED05 thresholds for occurrence, depth, and area.

The age-dependent pig threshold observation is interesting because it suggests that there may not be a monotonic threshold effect but rather an effect that depends on a specific age. It also suggests that neonate pigs have a low threshold that might impact the application of ultrasound with human neonates. Likewise, the oldest pigs studied also have a low threshold that might impact the application of ultrasound in older humans who are more likely to be subjected to echocardiographic studies. Based on the meager age-dependent database and lack of physical and biological mechanisms responsible for the onset of the ultrasound-induced lung hemorrhage, it is premature to suggest a hypothesis that might account for this nonmonotonic threshold effect. The original thought was to use three ages to determine whether there was an age-dependent threshold, and, if so, to identify the degree to which the threshold was monotonic. This thought was

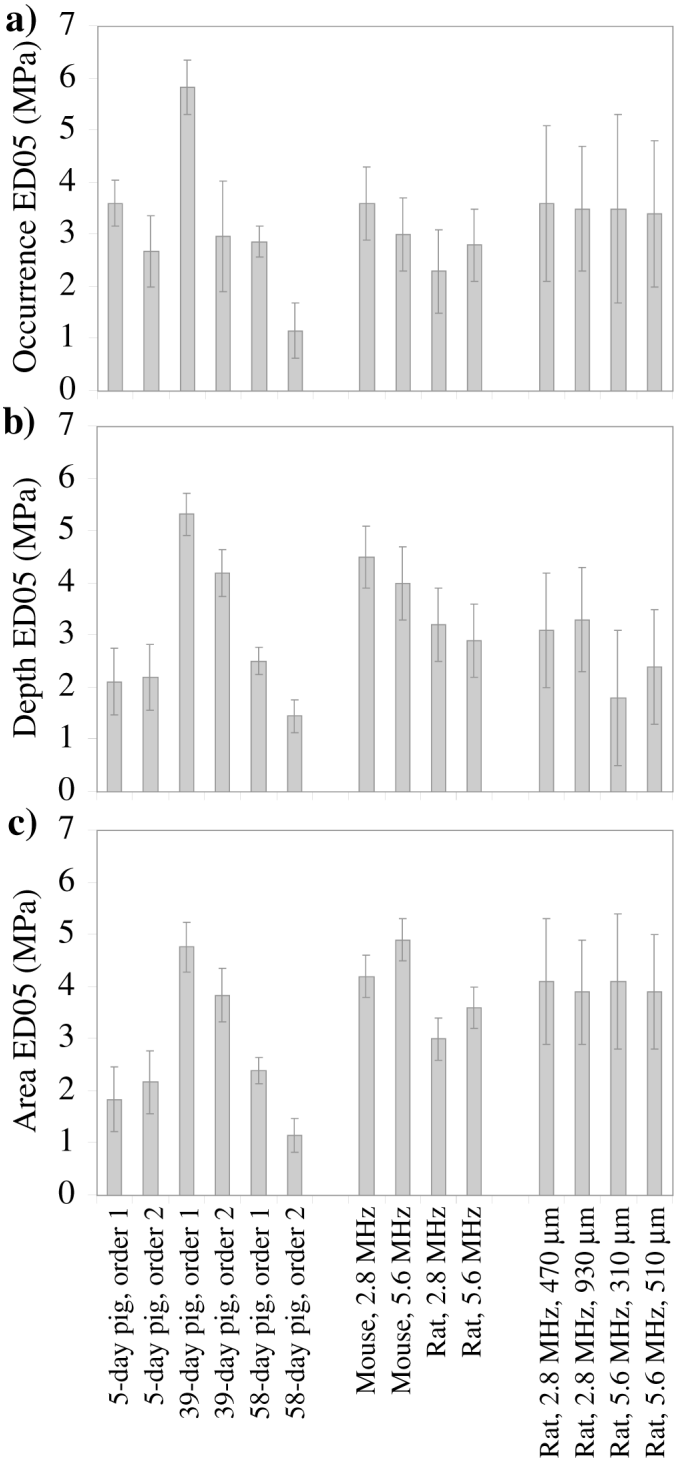

Fig. 5. Comparison of the ED05 threshold estimates of (a) lesion occurrence, (b) lesion depth, and (c) lesion surface area of the six pig age/order groups (six left bars) with the mouse/rat ED05 thresholds (four middle bars) [19], and the rat ED05 thresholds from the beamwidth study (four right bars) [21]. Error bars are the standard errors from each of these studies.

based on the fact that maturation changes as animals age are generally monototic [36]–[39]. Unfortunately, the findings reported herein yielded age-dependent thresholds that were not monotonic.

It is interesting to compare the pig ED05 thresholds to those of mice and rats for which the same experimental and data analysis procedures were used (Fig. 5). The only differences were frequency and species. The same 10-s exposure duration was used. The ED05 threshold estimates were made with 151 adult mice and 160 adult rats at two frequencies (2.8 and 5.6 MHz) in which the occurrence of lung damage was shown to be independent of both species and frequency [19]. The ED05 thresholds for lesion occurrence ranged between 3.0 and 3.6 MPa for the adult mice and between 2.3 and 2.8 MPa for the adult rats. The ED05 threshold estimates were made with 144 adult rats at the same two frequencies and at four beamwidths in which the occurrence of lung damage was shown to be independent of beamwidth [21]. The ED05 thresholds for lesion occurrence ranged between 3.4 and 3.6 MPa for the adult rats. These mice and rat ED05 threshold ranges overlapped the 5- and 58-day order 1 pigs (3.6 and 2.9 MPa, respectively); but they were lower than the 39-day order 1 pigs (5.8 MPa). Comparison here is made with the order 1 pigs because they are considered more consistent with the experimental protocol of the mice and rat studies (Fig. 6). Thus, the 39-day pigs appear to be less sensitive to the onset of lung damage compared to adult mice, adult rats, and the two other pig age groups.

However, based on lesion depth and area, the mice and rat ED05 thresholds (range: 1.8–4.9 MPa) generally were closer to the 39-day order 1 pig ED05 thresholds (range: 4.8–5.3 MPa) and higher than the 5- and 58-day order 1 pig ED05 thresholds (range: 1.8–2.5 MPa) (Fig. 5). This may be due to the trends in superthreshold effects, which influence the threshold estimates based on lesion depth and area measurements. It is an indication that the superthreshold effects in mice and rats are more similar to the 39-day pigs than to the neonate or 58-day pigs. The occurrence ED05 thresholds do not use superthreshold-size measurements, only the incidence rates. These thresholds, therefore, are less sensitive to differences in magnitude of effect as the exposure level increases.

Two separate studies by the same research group evaluated the lung damage threshold using 35 1-day-old crossbred pigs [10] and about 13 10-day-old crossbred pigs [14]. Both of these 2.3-MHz (10-μs pulse duration, 100-Hz PRF) studies yielded an estimated in situ peak rarefactional pressure threshold of about 0.9 MPa, substantially lower than we found with our mouse, rat, and pig studies. One difference is the methodology by which they estimated their threshold values. Their analysis used nonlinear least squares estimation of a piecewise linear model for the mean incidence rate, whereas our analysis used logistic and tobit analysis to estimate the probability of a lesion as a function of exposure. We define the threshold as the exposure leading to a 5% response probability; but, their analysis defines it as the point of inflection in the piecewise linear

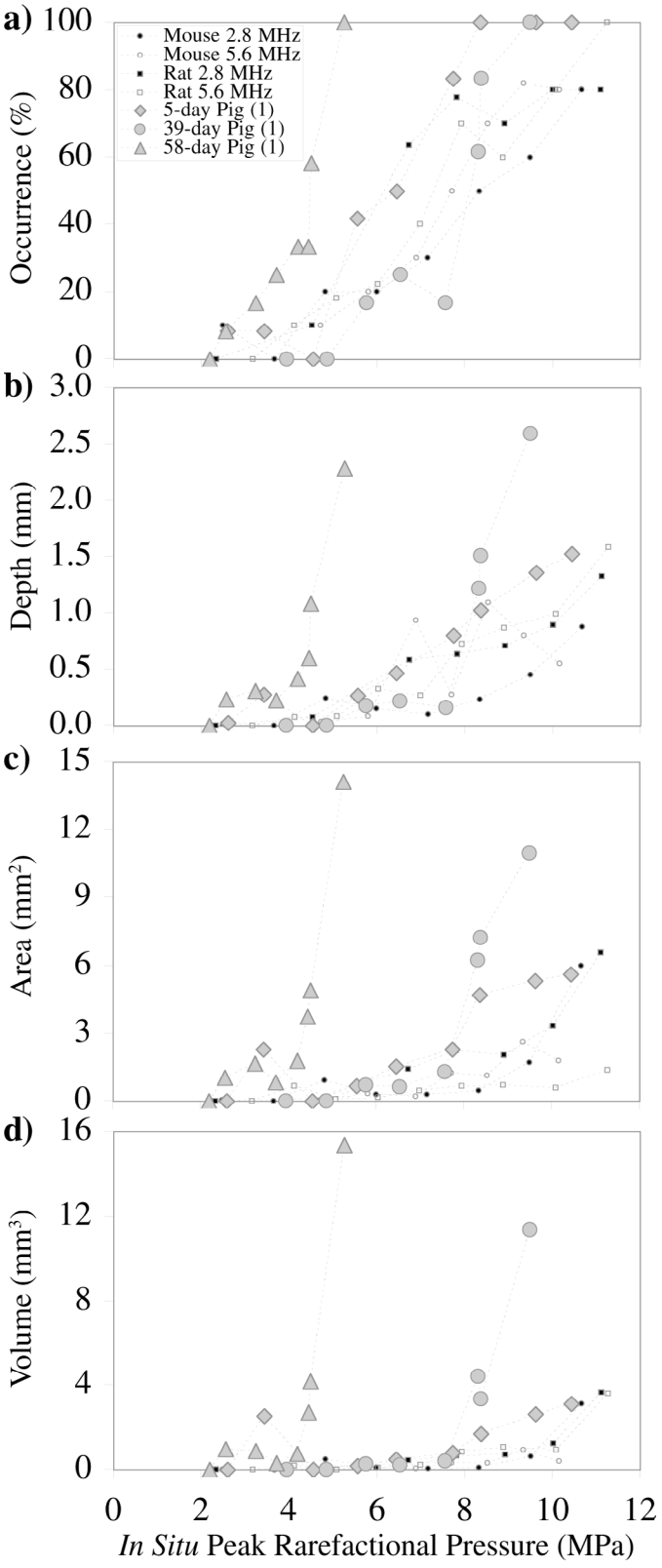

Fig. 6. Comparison of the (a) lesion occurrence, (b) lesion depth, (c) lesion surface area, and (d) lesion volume as a function of the in situ peak rarefactional pressure of three age order-1 pig groups (5-day, 39-day, and 58-day) with the mouse/rat threshold results [19]. The larger shaded symbols are the pig results; the smaller symbols are from the mouse/rat threshold results.

mean response function. When we apply our method to their data, we do not find major differences from their reported thresholds, however; so the lower thresholds appear to be due to the experimental procedure rather than the statistical analysis. In their exposure duration procedure, a 16-minute exposure duration procedure was used for the 1-day-old pigs [10] and a 16- and 20-minute exposure procedure was used for the 10-day-old pigs [14]. For each 16- or 20-minute exposure procedure, the same general region of the pleural surface was exposed in order to assure that the pig ribs did not block the sound beam from reaching the pleural surface. The pig was moved a distance of 2.5 mm every 2 minutes (source transducer did not move) while the ultrasound exposure continued for the 16 or 20 minutes. The −6-dB beamwidth at the focus of the 2.3 MHz transducer was 3 mm. Thus, 8 or 10 2-minute exposures separated spatially by 2.5 mm accounted for the 16- or 20-minute exposure procedure. Thus, it is likely that at each exposure site, one area could have received up to a 16- or 20-minute exposure because the normal-breathing pig's lung slides along the pleural surface a distance much greater than 2–3 mm. Also, the authors commented that the pig moved slightly. However, the authors have argued, but did not provide the basis for the argument, that the effective exposure duration at a given spot on the pig lung was 10 s. In reality, the exposure duration could be considered to be between 10 s and 16 or 20 minutes. If the exposure duration is, indeed, as long as 16 or 20 minutes, or at least much longer than our 10-s exposure duration, then this might explain their lower threshold (in situ peak rarefactional pressure was estimated to be 0.9 MPa).

An age-dependent lung damage threshold study evaluated 120 24- to 36-hour-old neonate, 120 14-day-old juvenile, and 60 8- to 10-week-old adult mice [13], and concluded that the estimated in situ peak rarefactional pressure thresholds for lesion occurrence were 0.6, 0.9, and 0.7 MPa, respectively, and that these thresholds were not significantly different. Exposure conditions were 1.15 MHz, 10-μs pulse duration, 100-Hz PRF and 3-minute exposure duration. Thus, an overall in situ peak rarefactional pressure threshold was 0.7 MPa. This age-independent mouse threshold agrees with the age-independent pig threshold [10], [14] from the same research group with a slight and probably insignificant difference in the reported threshold levels, that is, 0.7 MPa for mice and 0.9 MPa for pigs. What is interesting and counter to our findings is the reported threshold pressure levels that are determined from the lesion surface area. For the neonate, juvenile, and adult mice, the estimated in situ peak rarefactional pressure threshold levels are 1.2, 1.1, and 0.9 MPa, respectively. These threshold levels that were determined from lesion surface areas are greater than the threshold levels determined by lesion occurrence. In our pig studies, in all cases, the threshold levels determined from lesion surface area (and from lesion depth) are lower than the threshold levels determined by lesion occurrence. However, this observation is in agreement with our mouse and rat studies [19], [21], for which the threshold levels determined from

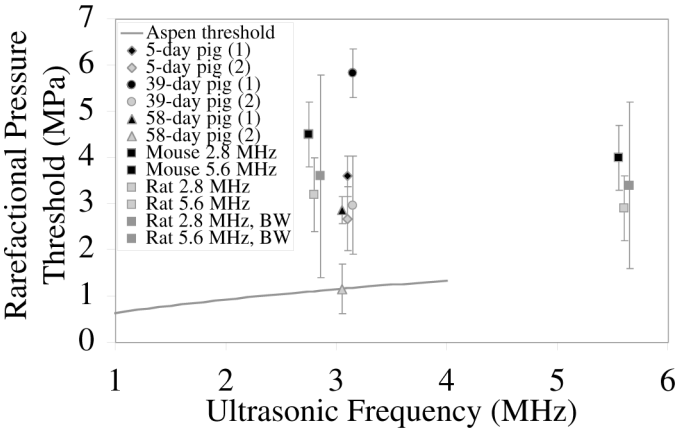

Fig. 7. Comparison of the ED05 rarefactional pressure threshold estimates of lesion occurrence of the six pig age/order groups with the four mouse/rat ED05 thresholds [19] and the two rat ED05 thresholds from the beamwidth study (BW) [21]. Error bars are the standard errors from each of these studies. The solid-line regression function ($0.63f^{0.54}$) is the derated rarefactional pressure threshold of ultrasound-induced lung damage derived from mouse, rat, and pig data [1].

lesion surface area are consistently greater than those determined from lesion occurrence. These differences may be due to lung type, suggesting that the interaction mechanism (physical and/or biological) may be a bit different and may need further examination.

The pig ED05 thresholds for lesion occurrence as well as the mouse and rat ED05 thresholds at both frequencies [19], [21] are shown graphically in Fig. 7 and compared against the regression function derived from the mouse, rat, and pig data that was analyzed in 1998 at the Aspen meeting [1]. The individual investigators reported their threshold observations (see Table 4-1 in [1]); they used different definitions for the onset of damage and different statistical treatments of their data. It is clear that the rarefactional pressure threshold values for the data reported by our group are greater than the data summarized at the Aspen meeting. This can be viewed as encouraging in terms of human health, for we are showing that the onset of lesion production is not as sensitive as previously viewed. However, the majority of the Aspen data was acquired with a longer exposure duration and a longer pulse duration than we have used. All of our data were acquired by using a 10-s exposure duration and a 1.2-μs pulse duration; the data summarized by the regression function were acquired by using an exposure duration of 180 s or greater and mostly a 10-μs pulse duration.

At superthreshold exposure conditions, a significantly different ED50 was observed as a function of age and showed that the oldest (58-day) pigs are more susceptible to lung damage than 5- and 39-day pigs given equivalent $p_{r(\text{in situ})}$ exposure (Table IV and Fig. 8). The six left bars in Fig. 8 graphically show the pig ED50s for occurrence, depth, and area. In all cases for all three measures (lesion occurrence, depth, and area), the oldest (58-day) pigs had significantly lower ED50s than 5-day and 39-day pigs.

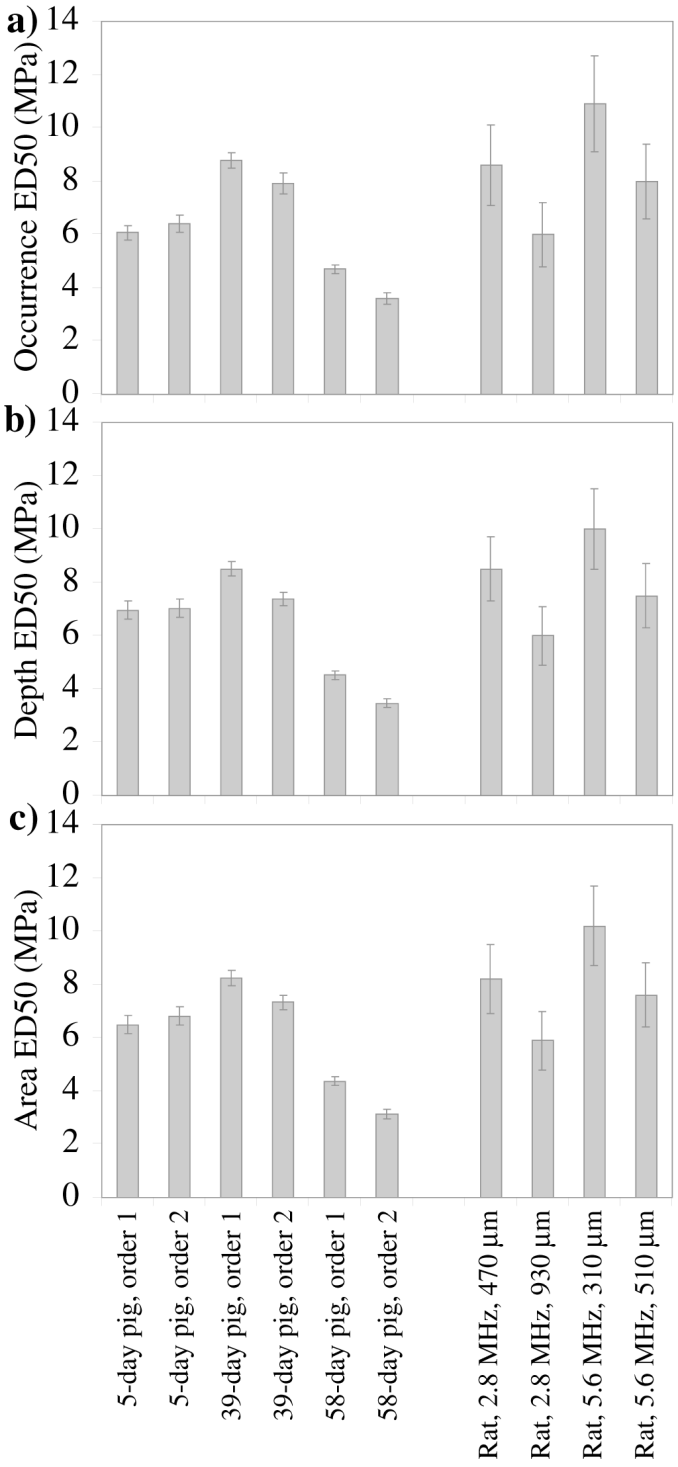

Fig. 8. Comparison of the ED50 estimates of (a) lesion occurrence, (b) lesion depth, and (c) lesion surface area of the six pig age/order groups (six left bars) with the rat ED50s from the beamwidth study (four right bars) [21]. Error bars are the standard errors from each of these studies.

It is interesting to compare the pig ED50 superthreshold levels to those of rats, for which the same experimental and data analysis procedures were used (Figs. 6 and 8). The differences were frequency and species. The same 10-s exposure duration was used. The ED50 estimates were made with 144 adult rats at two frequencies (2.8 and 5.6 MHz) and at four beamwidths in which the lung damage was shown to be dependent on beamwidth for all measures [21]. Therefore, comparison herein will be done against the 470- and 510-μm beamwidths, for which there was no significant difference and which are similar to the beamwidth used with the pig study (610 μm). The ED50s for lesion occurrence ranged between 8.0 and 8.6 MPa for the adult rats. This rat ED50 range was essentially the same as that of the 39-day order-1 pigs (8.8 MPa) but was greater than the 5- and 58-day pigs (6.1 and 4.7 MPa, respectively). Comparison here is made with the order-1 pigs because they are considered more consistent with the experimental protocol of the rat study. Thus, the oldest (58-day) pigs appear to be more sensitive to the growth of lung damage compared to adult rats, and the two other pig age groups.

For the order 1, and for all three measures, the oldest (58-day) pigs had significantly lower ED50s than the youngest (5-day) pigs, and the youngest (5-day) pigs had significantly lower ED50s than the 39-day pigs. This is somewhat consistent with the mouse superthreshold observation wherein the 24- to 36-hour-old neonate mice are the least sensitive to lesion growth, and the 8- to 10-week-old adult mice are most sensitive to lesion growth [13]. However, for the superthreshold mouse observations, the age-dependent results are monotonic (adult most sensitive, neonate least sensitive, and juvenile in between), whereas for our superthreshold pig observations, the sensitivity of the neonate (5-day) pig is in between that of the 58-day pig (most sensitive) and 39-day pig (least sensitive). There appears to be a difference in the lesion growth, either based on age or species; however, no study that we know of has evaluated this.

Order of exposure was also a significant factor in the threshold estimation. The second exposed lungs (order 2) had, in general, significantly lower ED05s and ED50s than the first exposed lungs (order 1). The only exceptions were for the depth and area ED05 thresholds and the ED50 values with the 5-day pigs. The down lung, because of gravity and pressure issues, is usually more poorly inflated [47]. This outcome is to be expected in animal and human patients while under anesthesia, and is an important concern in anesthetic protocols in human beings because of vertical to horizontal postural changes [47]–[49]. These postural changes cause alterations in normal respiratory and vascular dynamics in the lungs resulting in increased vascular perfusion and decreased pulmonary ventilation. Total lung capacity (TLC) and functional residual lung (FRL) volume are reduced in humans shifted from an erect to a supine posture; in fact, one study reported a 44% decrease in functional residual lung capacity in human patients in a lateral or prone position [48]. The larger (older) the pig, the more difficult was the alignment of the transducer, and thus the longer the animal was down. This may explain why the order of exposure effect became more pronounced as the size (and age) of the pig increased. An explanation for a difference seen between individual pigs is that the alignment procedure simply took longer in some pigs. In most cases, the alignment procedure was shorter in duration for the second exposed lungs because transducer positioning for the second side was similar to that for the first side. Therefore, very little transducer adjustment needed to be made on the second side. Hence, the second exposed lungs likely did not have adequate time to reinflate and, therefore, were in a more deflated state, compared to the first exposed lung, when exposed.

The degree of lung inflation appears to have a significant effect on the degree of ultrasound-induced lung damage. Our recent finding showed that the extent to which lung lesions in rats occur is inversely related to the acoustic impedance difference between intercostal tissue and lung; as lung inflation decreases, the incidence of lung hemorrhage and the lesion size increase [26]. In fact, when the rat lung was fully inflated, lung inflation volume was approximately 20% greater than tidal volume, no lesions were produced despite the fact that ultrasound exposure levels were so high that for normal-breathing rats, almost all of the lungs would have produced lesions. As the rat lung's inflation volume was decreased to 60% less than tidal volume, some lesions occurred; and, when the rat's lung was deflated, more lesions occurred, all with the same ultrasound exposure conditions. Thus, based on our inflation-dependent findings [26], the second exposed pig lung (the down lung during the first exposed lung, and the lung likely to be less inflated) would more likely be hemorrhaged than the first exposed pig lung. Also, the second exposed pig lung would more likely have a larger lesion than the first exposed pig lung. This then would result in a lower threshold for the second exposed pig lung because it would not be as fully inflated as compared to the first exposed pig lung.

The mechanical index (MI) thresholds, as assessed from the ED05 occurrence thresholds, were compared (Fig. 9) for the six pig age/order groups reported herein, the adult mouse and rat threshold study [19], and the adult rat beamwidth study [21]. The increase in the MI threshold as a function of pig age for order 1 exposures does not necessarily mean that the lungs of older pigs are less sensitive to damage because the MI is a water-based measurement that uses a constant derating factor. In fact, based on the in situ pressure thresholds (Fig. 5), the lungs of the order 1 oldest pigs are the most sensitive, thus showing that the MI can be misleading. The MI thresholds for the order 2 pigs clearly show a marked decrease relative to the order 1 pigs. All of the order 2 pigs are less than 1.9, the Food and Drug Administration's regulated limit [32], [33] of diagnostic ultrasound systems. This might suggest that, if a patient had some kind of a pulmonary disorder whereby the lungs were not normally inflated, there could be a risk of hemorrhage from a clinical diagnostic ultrasound exposure.

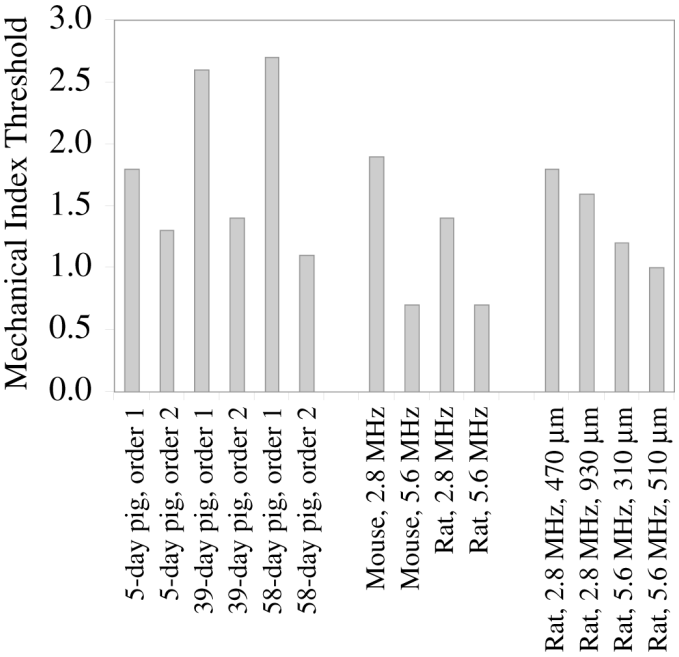

Fig. 9. Comparison of the MI thresholds, as assessed from the ED05 occurrence thresholds, of the six pig age/order groups (six left bars) with the mouse/rat study (four middle bars) [19], and the rat beamwidth study (four right bars) [21].

Comparison of the MI thresholds, based on occurrence of ultrasound-induced lung hemorrhage, among the three species evaluated (Fig. 9), shows that order 1 pigs are less sensitive to lung damage than the lungs of mice or rats. However, based on the in situ pressure thresholds (Fig. 5), there does not appear to be a difference except for the 39-day-old pig, suggesting that the youngest and oldest order 1 pigs have approximately the same threshold levels of damage as those for mice and rats. This is consistent with previous observations for pigs less than 10-days old [10], [14]; but the threshold levels reported herein are higher than those previously reported.

V. Summary

It has been shown that there is an age-dependent effect of ultrasound-induced lung damage in pigs, although the in situ pressure threshold levels do not change monotonically with age. The middle-aged (39-day-old) pigs were the least sensitive to lung damage relative to the youngest (5-day-old) and oldest (58-day-old) pigs. It also was shown that lung inflation appears to play an important role in determining the threshold for lung damage.

Acknowledgments

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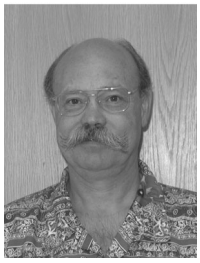

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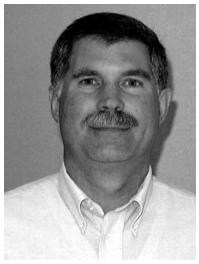

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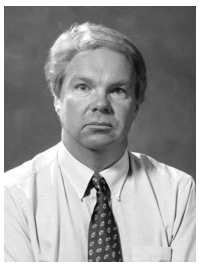

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