Age-dependent Threshold and Superthreshold Behavior of Ultrasound-induced Lung Hemorrhage in Pigs

William D. O'Brien Jr.,[1] Douglas G. Simpson,[2] Leon A. Frizzell,[1] and James F. Zachary[3]
[1]Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois, 405 North Mathews, Urbana, IL 61801; [2]Department of Statistics, University of Illinois, 725 S. Wright Street, Champaign, IL 61820; and [3]Department of Veterinary Pathobiology, University of Illinois, 2001 S. Lincoln Avenue, Urbana, IL 61802

Abstract - Age-dependent threshold and superthreshold behavior of ultrasound-induced lung hemorrhage was investigated with 116 4.9±1.6-day-old neonate crossbred pigs, 103 39±5-day-old crossbred pigs, and 104 58±5-day-old crossbred pigs. Exposure conditions were: 3.1 MHz, 10-s exposure duration, 1-kHz PRF, and 1.4-μs pulse duration. The *in situ* (at the pleural surface) peak rarefactional pressure ranged between 2.2 and 10.4 MPa with either 8 or 9 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 and right lung) and acoustic pressure both randomized. Logistic regression analysis was used to examine the dependence of the lesion incidence rates on *in situ* peak rarefactional pressure, left versus right lung, order of exposure (first versus second) and age in three categories. Likewise, lesion depth and 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. Oldest pigs had significantly lower thresholds (2.63±0.27 MPa) than middle-aged pigs (4.84±0.49 MPa). The oldest pigs also had lower thresholds than neonate pigs (3.62±0.44 MPa). Overall the oldest pigs were most sensitive to lung damage, and neonates were more susceptible than middle-aged pigs. 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 threshold occurrences: 3.62 to 2.67, 4.84 to 2.23 and 2.63 to 1.04 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 in the thresholds.

I. Introduction

Diagnostic ultrasound is one of the most widely used and safest imaging modalities available in medical practice. Concerns for its safety have recently been raised and addressed by members of the bioeffects research community [1-3]. These concerns are the result of a number of ultrasound-induced lung hemorrhage studies in mice, rats, rabbits, monkeys and pigs.

Two crossbred pig studies by the same group using neonate (1-day-old) [4] and young (10-day-old) [5] 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 [1]. This difference suggested an age-dependent effect. Therefore, the study reported herein estimated the *in situ* peak rarefactional pressure threshold levels in crossbred pigs at three ages, 4.9±1.6-, 39±5-, and 58±5-day-old.

II. Animals

The experimental protocol was approved by the campus' Laboratory Animal Care Advisory Committee and satisfied all campus and NIH rules for the humane use of laboratory animals. Animals were housed in an 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*.

There were a total of 116 4.9±1.6-day-old neonate crossbred pigs (2.1±0.3 kg), 103 39±5-day-old crossbred pigs (9.8±1.1 kg), and 104 58±5-day-old crossbred pigs (20±1.2 kg). 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 8 (39-day

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and 58-day) or 9 (5-day) acoustic pressure levels, each with 12 pigs/pressure level (except one of the 39-day pig groups that had 13 pigs/pressure level). Of the total 323 pigs, eight 5-day pigs, six 39-day pigs and eight 58-day pigs were shams, and were included in the randomized design; no lesions were produced in the sham exposed pigs.

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, followed by a depilatory agent (Nair: Carter-Wallace, Inc., New York, NY) to maximize sound transmission. Anesthetized pigs were placed in right or left lateral recumbancy and a stand-off tank positioned in contact with the skin. Mineral oil was used as the coupling agent on the skin surface. All pigs were exposed bilaterally. The ultrasound beam was approximately perpendicular to the skin, between the fifth and sixth ribs, 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 aligned again, 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, or right then left lung) was randomized with equal exposures first to left lung (second to right lung) and first to right lung (second to left lung).

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 since, 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).

The lungs were removed from each pig, rinsed in 0.9% sodium chloride, examined grossly for the presence or absence of a lesion and 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 and was fixed by immersion in 10% neutral-buffered formalin for a minimum of 24 hours. After fixation, the elliptical dimensions of lung lesions at the visceral pleural surface were measured with a digital micrometer, where "a" was the length of the semi-major axis and "b" was the length of the semi-minor axis. The lesions were then bisected and the depth "d" of the lesion within the lung was measured. In animals where the depth of the lesion was not visually discernable, the depth was determined from measurements made on histologic sections with a slide micrometer. The surface area (πab) and volume ($\pi abd/3$) of the lesion were calculated for each animal. Each half of the bisected lesion was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and evaluated microscopically.

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.

III. 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 [3].

An automated procedure that was based on established standards [6] was used to routinely calibrate the ultrasound fields [3], and yielded the peak *in vitro* (water-based) rarefactional pressure and the peak *in vitro* compressional pressure, as well as the Mechanical Index [6]. The *in situ* (at the pleural surface) peak rarefactional and compressional pressures were estimated for each pig from

$$p_{r(\text{in situ})} = p_{r(\text{in vitro})} e^{-(Ax)}$$

and

$$p_{c(\text{in situ})} = p_{c(\text{in vitro})} e^{-(Ax)}$$

The mean attenuation coefficient, A, of the pig's intercostal tissue 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 [7].

IV. Statistics

Logistic regression analysis was used to examine the dependence of the lesion incidence (occurrence) rates on *in situ* peak rarefactional pressure [8]. 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) [3,9]. The ED05 level will be referred to as the ED05 threshold. Depth and root surface area of lesions were analyzed using Gaussian tobit regression to give similar ED05 and ED50 levels [10,11].

V. Results and Discussion

The mixed model analysis did not detect a significant correlation between incidence (occurrence) of lesions on the two sides after adjusting for *in situ* peak rarefactional pressure, 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 applied to the results of lesion occurrence (Fig. 1) 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.

The occurrence differences between 5-day pig ED05 thresholds and 58-day ED05 thresholds were not statistically significant (Fig. 2). The 39-day pigs had significantly higher ED05 thresholds than the 5-day and 58-day pigs. 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 differences between 5-day pig ED05 depth thresholds and 58-day ED05 depth thresholds were not statistically significant, and the 39-day pigs had significantly higher ED05 depth thresholds than the 5-day and 58-day pigs for both orders 1 and 2 (Fig. 2). 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 ED05 surface area thresholds for 5-day and 58-day pigs were not statistically significant, and the 39-day pigs had significantly higher ED05 surface area thresholds than both 5-day and 58-day pigs for both orders 1 and 2 (Fig. 2). 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.

Overall, the oldest pigs were most sensitive to lung damage, and neonates were more susceptible than middle-aged pigs. The surprising sensitivity of oldest pigs to ultrasound damage necessitates further study of the age dependence of ultrasonic lesion threshold levels.

Order of exposure was also a significant factor in the threshold estimation. The lung exposed second (order 2) had, in general, significantly lower ED05 thresholds than the first lungs exposed (order 1). Occurrence ED05 threshold levels for order 1 and order 2, respectively, were: 3.60±0.4 and 2.68±0.7 (neonates), 5.83±0.5 and 2.97±1.1 (middle-aged), and 2.87±0.3 and 1.16±0.5 MPa (oldest). Note that there was a significant decrease in the threshold occurrences, changes that are greater that the confidence limits, for all age groups. Thus, a subtle effect in lung physiology, and a major effect in the thresholds were observed.

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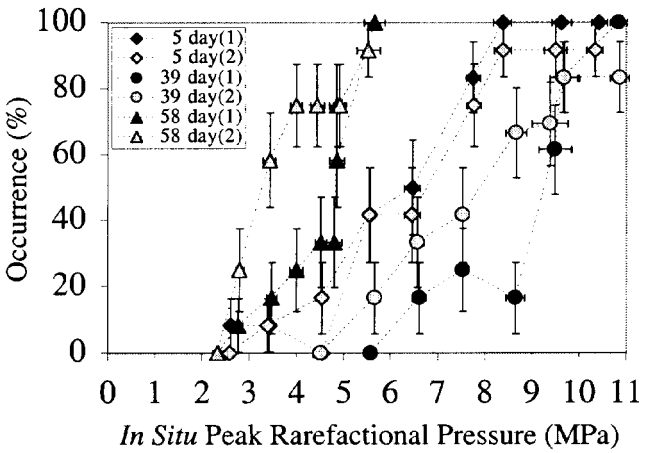

Fig. 1. Lesion occurrence as a function of the *in situ* peak rarefactional pressure. The dashed lines are straight lines connecting the mean values. For each age group (5-day, 39-day and 58-day pigs), there are two data sets, one representing the first exposed lungs (order 1), denoted by (1), and the other representing the second exposed lungs (order 2), denoted by (2).

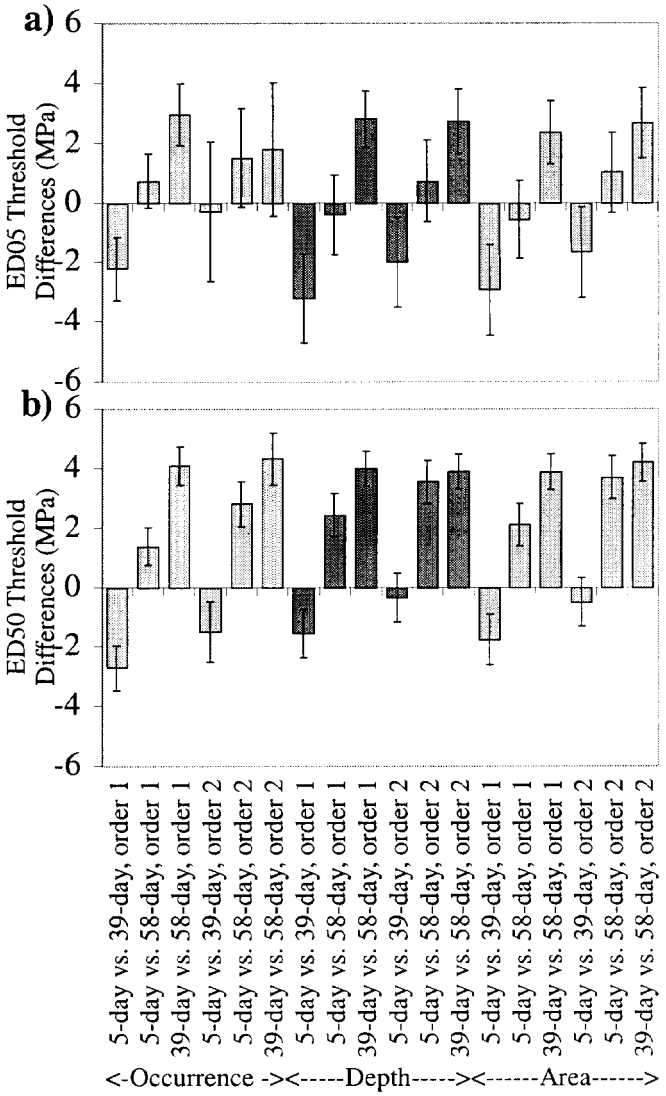

Fig. 2. Paired comparisons of age-specific ED05 threshold (a) and ED50 level (b) differences for occurrence, depth and surface area of lesions. A positive difference indicates that the first listed pig age has a greater value than the second listed pig age. The confidence bars are the ±1.96 standard errors; a difference is statistically significantly if the confidence bar does not cross the zero-difference axis.