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● *Original Contribution*

THRESHOLD ESTIMATION AND SUPERTHRESHOLD BEHAVIOR OF ULTRASOUND-INDUCED LUNG HEMORRHAGE IN RATS: ROLE OF AGE DEPENDENCY

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Abstract—Age-dependent threshold and superthreshold behaviors of ultrasound-induced lung hemorrhage were investigated with one hundred ten 12.6 ± 0.8-d-old rats, one hundred ten 22.9 ± 0.8-d-old rats, and one hundred 57.7 ± 3.9-d-old rats. Exposure conditions were: 2.8 MHz, 10-s exposure duration, 1-kHz pulse repetition frequency and 1.3-μs pulse duration. The *in situ* (at the pleural surface) peak rarefactional pressure ($p_{r(in\ situ)}$) ranged between 1.4 and 10.8 MPa for which there were either 9 or 10 acoustic pressure groups for each of the three rat ages (10 rats/exposure group). For each of the three rat ages there were also shams; there were no lesions in the shams. The $p_{r(in\ situ)}$ levels were randomized within each age group; rat age was not randomized. Individuals involved in animal handling, exposure and lesion scoring were blinded to the exposure condition. In addition, one hundred fifty-six 72-d-old rats were included from three completed studies (same experimental conditions) to provide a fourth age group for the analysis. Probit regression analysis was used to examine the dependence of the occurrence of lesions on $p_{r(in\ situ)}$ in the four age groups. Likewise, lesion depth and lesion root surface area were analyzed using Gaussian tobit regression analysis. Although $p_{r(in\ situ)}$ was a significant variable, no significant age dependence of the $p_{r(in\ situ)}$ effect was found. Furthermore, age had no significant effect on either the rate of occurrence or the depth of lesions. Given the occurrence of a lesion, a weak age dependence was found for the median surface area of the induced lesion (*p*-value = 0.037). (E-mail: wdo@uiuc.edu) © 2008 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Lung, Pulmonary hemorrhage, Pulsed ultrasound, Rat.**

INTRODUCTION

The study reported herein evaluates one of the risk factors for ultrasound-induced lung hemorrhage, that is, age dependency because there have been several conflicting observations. These conflicting observations have been summarized previously (O'Brien et al. 2003a). Briefly, monkey studies suggested that younger animals had a greater likelihood for ultrasound-induced lung hemorrhage compared with older animals (Tarantal and Canfield 1994). Mice were reported to have an age-independent ultrasound-induced lung-hemorrhage threshold (Dalecki et al. 1997a). Also, one research group reported that neonate (1-d-old) (Baggs et al. 1996) and young (10-d-old) pigs had an age-independent lung-hemorrhage threshold (Dalecki et al. 1997b). However, when these younger (1- and 10-d-old) pig studies are compared with another pig study of older animals (10- to 12-wk-old) (O'Brien and Zachary 1997), younger pigs were more sensitive than the older pigs, that is, an age-dependent observation between separately conducted studies. And, age-dependent lung-hemorrhage thresholds were reported with middle-aged (39-d-old) pigs being least susceptible to lung damage, oldest (58-d-old) pigs being most susceptible to lung damage and neonate (4.9-d-old) pigs having intermediate susceptibility to lung damage (O'Brien et al. 2003a).

Three species (mouse, pig, and monkey) have been investigated but the age dependencies of ultrasound-induced lung hemorrhage in rats have not. 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 Sprague Dawley rats at three ages (12.6 ± 0.8-d-, 22.9 ±

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0.8-d-, 57.7 ± 3.9-d-old). In addition, a fourth age group (72-d-old) was included for the age-dependent ultrasound-induced lung hemorrhage analysis from a compilation of three completed rat studies (Zachary et al. 2001; O'Brien et al. 2001, 2003b) for which the same experimental conditions were used.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois and satisfied all campus and National Institutes of Health rules for the humane use of laboratory animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), Rockville, MD -approved animal facility and provided food and water *ad libitum*.

There were a total of one hundred ten 12.6 ± 0.8-d-old (mean ± SD) rats (weight: 26.7 ± 3.6 g; chest wall thickness: 1.6 ± 0.2 mm), one hundred ten 22.9 ± 0.8-d-old rats (weight: 53.4 ± 6.6 g; chest wall thickness: 1.8 ± 0.1 mm), and one hundred 57.7 ± 3.9-d-old rats (weight: 235 ± 48 g; chest wall thickness: 3.8 ± 0.5 mm). All Sprague Dawley rats were obtained from Harlan (Indianapolis, IN, USA). These three age groups will also be referred to as 13-d-, 23-d- and 58-d- rats for convenience. 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 rat time points. These time points are linked to biological events. Such categories include preweaned rats (13-d-old group), postweaned rats (23-d-old group) and adult rats (58-d-old group).

In each age group, rats were assigned randomly to 11 (13 d and 23 d) or 10 (58 d) acoustic pressure levels (Table 1), each with 10 rats/pressure level. Pulsed ultrasound exposure conditions were: 2.8 MHz, 10-s exposure duration, 1-kHz pulse repetition frequency and 1.3-μs pulse duration. No lesions were produced in the sham exposed rats. The individuals involved in rat handling, exposure, necropsy and lesion scoring were blinded to the exposure condition. The exposure condition for each rat was revealed only after the final results were tabulated.

In addition, a fourth age group (156 72-d-old rats) was included for the age-dependent ultrasound-induced lung hemorrhage analysis from a compilation of three completed studies (Zachary et al. 2001; O'Brien et al. 2001, 2003b) for which the same experimental conditions were used.

Rats were weighed and then anesthetized with ketamine hydrochloride (87.0 mg/kg) and xylazine (13.0 mg/kg) administered intraperitoneally. Hair of the left thorax was removed with an electric clipper, followed by a depilatory agent (Nair® Carter-Wallace, Inc., New York, NY, USA) to maximize sound transmission. A black dot was placed at approximately the sixth to ninth rib to guide the positioning of the ultrasonic beam. Anesthetized animals were placed in a specially designed transducer holder. A removable pointer, attached to the transducer, was used to position the ultrasonic beam perpendicular to the skin at the position of the black dot with the beam's focal region approximately at the lung surface. The holder with animal and mounted transducer was placed in highly degassed, temperature-controlled (30°C) water. In pulse-echo mode, the low-power output signal (see shams in Table 1) from the RAM5000 (Ritec, Inc., Warwick, RI, USA) was displayed on an oscilloscope and used to adjust the axial center of the focal region to within 1 mm of the lung surface. Following exposure, animals were removed from the water and holder, and then euthanized under anesthesia by cervical dislocation.

The thorax was opened and the thickness of each left thoracic wall (skin, rib cage and parietal pleura) was measured using a digital micrometer (accuracy: 10 μm; Mitutoyo Corp., Kawasaki, Kanagawa, Japan) to calculate the *in situ* peak rarefactional pressure. The left lung lobe was scored for the presence or absence of hemorrhage and then fixed by immersion in 10% neutral-buffered formalin for a minimum of 24 h. After fixation, the elliptical dimensions of each lung lesion at the visceral pleural surface were measured using a digital micrometer where "a" is the semi-major axis and "b" is the semi-minor axis. The lesion was then bisected and the depth "d" of the lesion within the pulmonary parenchyma was also measured. The surface area (πab) of the lesion was 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.

Exposimetry

The exposimetry and calibration procedures have been described previously in detail (Zachary et al. 2001). Ultrasonic exposures were conducted using a focused f/1 19-mm-diameter, lithium niobate ultrasonic transducer (Valpey Fisher, Hopkinton, MA, USA). Water-based (highly degassed water, 22°C) pulse-echo ultrasonic field distribution measurements were performed according to established procedures (Raum and O'Brien 1997) and