Effect of Contrast Agent on the Incidence and Magnitude of Ultrasound-Induced Lung Hemorrhage in Rats

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Objective: To test the hypothesis that inertial cavitation in the vasculature of the lung is not the physical mechanism responsible for ultrasound-induced lung hemorrhage. Methods: A factorial design was used to study the effects of two types of injected agents (IA; 0.25 ml per rat of saline or Optison® given intravenously) and two levels of pulsed ultrasound exposure (UE; in situ peak rarefactional pressures of 2.74 and 5.86 MPa; respective mechanical indices of 1.02 and 2.14) on the incidence and size of lung lesions. Ten 10-to-11-week-old Sprague-Dawley rats were exposed to pulsed ultrasound at each of the four combinations of IA and UE at a center frequency of 3.1 MHz, exposure duration of 10 s, pulse repetition frequency of 1,000 Hz and pulse duration of 1.2 μs. In addition, nine rats served as shams in which no lung hemorrhage occurred. Results: Rats administered contrast agent prior to exposure did not have an increase in lesion occurrence or size compared to rats that received saline with no contrast agent. Conclusions: These results provide further evidence that the mechanism for production of lung hemorrhage is not inertial cavitation. (ECHOCARDIOGRAPHY, Volume 21, July 2004)

lung hemorrhage, Optison®, contrast agents, bioeffects, pulsed ultrasound

It has been suggested that inertial cavitation is the mechanism responsible for lung hemorrhage in mice,[1] rats,[2] and monkeys.[3] Recently, however, some investigators have published data that support an argument that inertial cavitation in the vasculature is not responsible for lung damage,[4–6] and results of an overpressure study on mice presented evidence that inertial cavitation may not be involved in ultrasonically induced damage to lung.[7] These experimental observations have led to a continued scientific debate about whether inertial cavitation is responsible for the ultrasound-induced lung hemorrhage.[8–11]

The purpose of this study was to determine if an intravenous contrast agent would increase the severity of lung hemorrhage when the lung was exposed to ultrasound. These results will provide additional experimental evidence regarding the role of inertial cavitation in ultrasound-induced lung hemorrhage.

Materials and Methods

Exposimetry

Ultrasound exposure (UE) conditions, transducer characterization, and calibration procedures used in this study were described in detail previously.[12–16] A focused 51-mm diameter, lithium niobate ultrasonic transducer (Valpey Fisher, Hopkinton, MA, USA) was used to expose each lung. The pulse-echo characterization procedure yielded a center frequency of 3.1 MHz, a fractional bandwidth of 15%, a focal length of 56 mm, a −6-dB focal beamwidth of 610 μm, and a −6-dB depth of focus of 5.9 mm. The calibration procedure used a calibrated PVDF hydrophone (Marconi Model Y-34-6543, Chelmsford, UK) and yielded in vitro peak rarefactional pressures of 3.26 and 6.97 MPa (with in vitro peak compressional pressures of 3.75

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and 10.6 MPa, and mechanical indices of 1.02 and 2.14, respectively) for the two UE conditions. The pulse duration was 1.2 μs and the pulse repetition frequency was 1 kHz. The in situ (at the pleural surface) acoustic pressures were estimated by taking into account the attenuation of interposed intercostal tissue (1.1 dB/cm per MHz),[17,18] thus yielding in situ peak rarefactional pressures of 2.74 and 5.86 MPa (with in situ peak compressional pressures of 3.16 and 8.91 MPa, respectively). The respective in situ pulse-average intensities were 185 and 998 W/cm^2, and the respective in situ temporal-average intensities were 0.22 and 1.2 W/cm^2.

Animals

The experimental protocol was approved by the campus' Laboratory Animal Care Advisory Committee and satisfied all campus and National Institutes of Health rules for the humane use of laboratory animals. A total of 49 10-to-11-week-old 269 ± 15-g female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were used in this study. The 49 rats were randomly divided into seven groups: 10 rats were injected with Optison® and exposed at the lower pressure level, 10 rats were injected with saline and exposed at the lower pressure level, 10 rats were injected with Optison® and exposed at the higher pressure level, 10 rats were injected with saline and exposed at the higher pressure level, 3 rats were injected with Optison® and not exposed, 3 rats were injected with saline and not exposed, and 3 rats were not injected with anything and not exposed. The latter three groups of 3 rats each served as shams; there were no lung lesions in these 9 rats.

Rats were weighed and anesthetized with ketamine hydrochloride (87.0 mg/kg) and xylazine (13.0 mg/kg) administered intraperitoneally. The skin over the left thorax was shaved and depilated to maximize sound transmission. An intravenous 25-gauge catheter was inserted into the lateral tail vein and taped in place. A black dot was placed on the skin over the ribs at approximately the sixth to ninth rib to guide the positioning of the ultrasonic beam. A standoff tank was positioned in contact with the skin using mineral oil as a coupling agent. A circular transducer holder was visually centered above the black dot. The transducer was placed in the holder in the standoff tank that contained highly degassed water at 30°C. The low power pulse-echo capability of the exposure system

(RAM5000, Ritec, Inc., Warwick, RI, USA) displayed on a digital oscilloscope (LeCroy Model 9354CTM, Chestnut Ridge, NY, USA) was used to adjust the axial center of the focal region to within 1 mm of the lung surface. The ultrasonic beam axis was approximately perpendicular to the lung surface at the position of the black dot with the beam's focal region on the pleural surface.

For two of the 10-rat groups and one of the 3-rat sham groups, a single bolus of Optison® was injected intravenously (0.25 ml total dose) into the lateral tail vein of the 269-g rats followed by 0.25 ml of 0.9% NaCl. From a stock solution of Optison® (about $(5–8) \times 10^8$ microspheres/ml),[19] the 0.25-ml bolus contained about $(1–2) \times 10^8$ microspheres. Normalizing the microsphere concentration to the rat's body weight, the Optison® concentration was about 4×10^8 microspheres/kg (Table I). Based on body weight, this Optison® dose exceeded the manufacturer's recommended dose for use in echocardiography by 8–143 times.[20,21]

For the other two 10-rat groups and one of the 3-rat sham groups, a single bolus of 0.25-ml 0.9% NaCl was injected intravenously, followed by 0.25 ml of 0.9% NaCl.

The same Optison® dose injected in the same manner in rats of the same size was previously used in our study that demonstrated electrocardiogram arrhythmias in rat hearts exposed to ultrasound following injection of the contrast agent.[15] In this study and in the arrhythmia study,[15] UE of 3.1 MHz was commenced within about 1 minute after the bolus injection of Optison®. The arrhythmia study demonstrated that Optison® was circulating in the pulmonary vasculature because of the nature of the electrocardiogram arrhythmias, and also demonstrated that ultrasound effectively interacted with the contrast agent.

Following UE, rats were euthanized under anesthesia, the lungs were removed from the chest, and the chest wall thickness was measured (used later for calculation of the in situ ultrasonic pressures). The lungs were fixed by immersion in 10% neutral-buffered formalin, and the dimensions of lung lesions were measured with a Mitutoyo (Kawasaki, Kanagawa, Japan) 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. The surface area (πab) and volume

TABLE I

Comparison of Contrast Agents and Exposure Quantities Between the two Previous Studies that Examined Ultrasound-Induced Lung Hemorrhage Using Contrast Agent[5,25] and This Study

	Dalecki et al.[25]	Raeman et al.[5]	This Study
Animal	35-g mouse	35-g mouse	269-g rat
Contrast agent	Albunex®	Albunex®	Optison®
Max contrast agent concentration (microspheres/kg)	$1.4 \times 10^{9*}$	1.4×10^9	4×10^8
$p_{r(\text{in vitro})}/p_{c(\text{in vitro})}$ (MPa)	NR/2	1.6/2	3.26/3.75 & 6.97/10.6
$p_{r(\text{in situ})}/p_{c(\text{in situ})}$ (MPa)	NR/NR	1.4/1.7	2.74/3.16 & 5.86/8.91
Center frequency (MHz)	0.1	1.15	3.1
Pulse duration (μs)	15	10	1.2
Pulse repetition frequency (Hz)	1	100	1000
Exposure duration (s)	200	300	10
Number of pulses	200	30,000	10,000

NR: not reported.
*A 35-g mouse is assumed.

($\pi abd/3$) of the lesion were calculated for each animal.

The individuals involved in animal handling, exposure, and lesion scoring were blinded to the injected agent (IA) and exposure condition. The IA and exposure condition for each animal were revealed only after the final results were tabulated.

Statistical Methods

A factorial design was used to study the effects of IAs (saline vs Optison®) and UEs (level 1: $p_{r(\text{in situ})} = 2.74$ MPa; level 2: $p_{r(\text{in situ})} = 5.86$ MPa) on the occurrence and size of lung lesions. Fisher's exact test[22] was used to test for association between IA and lesion occurrence, controlling for UE. A censored rank test[23] was used to test for association between IA and lesion size (depth and surface area), also controlling for UE. The exact conditional significance levels of the tests were computed using the SAS procedures FREQ and NPAR1WAY.[24]

Fisher's exact analysis of lesion incidence was performed in two stages. First, Table II was constructed, which shows the presence or absence of lesions versus the four treatment combinations (saline at 2.74 MPa, Optison® at 2.74 MPa, saline at 5.86 MPa, Optison® at 5.86 MPa). Fisher's exact test was performed for this combined table to determine whether there was an association between lesion occurrence and the combination of UE level and IA. If the test was significant, Fisher's exact test was then performed in each of the 2×2 subtables for lesion occurrence by IA, controlling for UE. Letting p* denote the smaller of the two P-values from the subtables, the combined P-value was computed as $p = p^* (2 - p^*)$, which follows from standard formulas for combining results of independent tests. The test based on subtables was used to determine whether there was an association between lesion occurrence and the injected agent after adjustment for the UE.

In the Wilcoxon-Mann-Whitney censored rank analysis of lesion size (depth and surface area), the two-sample rank statistic (Optison® vs saline) was computed for each level of UE. Because a substantial percentage of animals exhibited no lesions (lesion size = 0), the censored version of the rank test was used.[23] The exact significance levels (P-values) of the censored rank statistics were evaluated using the SAS procedure NPAR1WAY,[24] which allows for ties in the ranks. The P-values for the two levels of ultrasound were combined as described above

TABLE II

Lesion Incidence Versus UE and IA

UE	IA	Lesion Presence	
		Yes	No
2.74 MPa	Saline	1	9
2.74 MPa	Optison®	0	10
5.86 MPa	Saline	4	6
5.86 MPa	Optison®	4	6
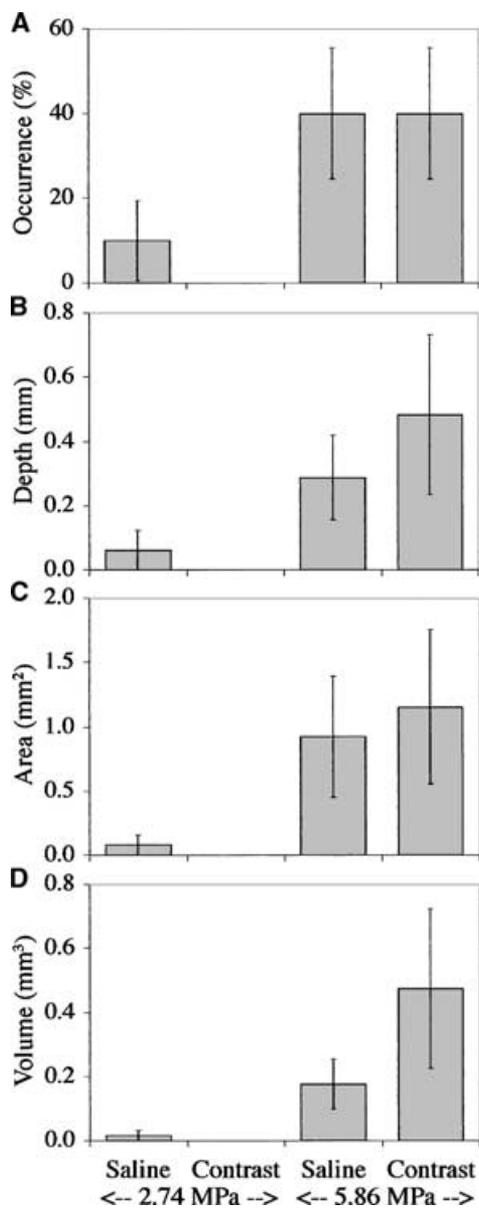
Figure 1. *Rat lung hemorrhage* **(A)** *occurrence,* **(B)** *depth,* **(C)** *surface area, and* **(D)** *volume of the four IA (saline and Optison®) and UE (in situ peak rarefactional pressures of 2.74 and 5.86 MPa) groups, each showing the mean and ± standard error of the mean (10 rats per group).*

to provide an overall test of significance of the contrast agent effect on lesion size.

Results

Table II and Figure 1A show the numbers of animals with and without lesions for each combination of IAs and UE. At the lower UE level, only one lesion was observed. Lesions were more common at the higher UE level.

Fisher's exact analysis of lesion occurrence is summarized in Table III. The test based on the 4×2 table indicates that there is a significant dependence of lesion occurrence on the combination of ultrasound level and IA. The two tests of conditional association between IA and lesion occurrence had P-values of 0.5, giving an overall P-value of $(0.5) (2 - 0.5) = 0.75$, so there is no significant effect of IA after adjustment for UE. It follows that the significant effect in the 4×2 table must be due to UE. This was confirmed by computing the Fisher exact test for UE, combining lesion counts over the two IAs. The significance of the UE effect confirmed that the experiment has adequate power to detect a known effect.

The censored rank analyses of lesion depth and lesion surface area (Fig. 1B and C) are summarized in Table IV. The rank tests of association between IA and lesion depth, conditional on UE, gave P-values of 0.50 and 0.41. These combine to give an overall P-value of $(0.41) (2 - 0.41) = 0.65$, so that there is no significant effect of IA on lesion depth after adjustment for UE. The conditional rank tests of association between IA and lesion surface gave P-values of 0.50 and 0.48, for an overall P-value of $(0.48) (2 - 0.48) = 0.73$, so there is no significant effect of IA on lesion surface area after adjustment for UE.

Lesion volume was not evaluated statistically because it is not an independent variable, but is provided graphically (Fig. 1D) for completeness.

Discussion

The results reported herein show that contrast agent per se did not increase the effect on lesion occurrence or size (depth and surface area) after adjustment for UE. Our results are consistent with those of Raeman et al.,[5] who evaluated the same hypothesis as we did, but in 35-g mice using Albunex® as the cavitation nuclei. Our investigation used a different species (rat) that was much larger (269 g) and a different cavitation nucleus (Optison®). The center frequency used by Raeman et al.[5] (1.1 MHz) was lower than ours (3.1 MHz), the exposure duration (300 s) was greater than ours (10 s), the pulse repetition frequency (100 Hz) was lower than ours (1 kHz), and the pulse duration (10 μs) was greater than ours (1.2 μs). Also, the in situ peak rarefactional pressure (1.4 MPa) was lower than both of ours (2.74 and 5.86 MPa).

TABLE III

Fisher Exact Analysis of Lesion Occurrence

Null Hypothesis	Conditioning Variables	Frequency Table	One-Sided P-Value for Exact Test
Lesion occurrence independent of UE and IA*	—	4 × 2 (Table II)	0.031
Lesion occurrence conditionally independent of IA	UE = 2.74 MPa	2 × 2 (Table II, rows 1–2)	0.50
	UE = 5.86 MPa	2 × 2 (Table II, rows 3–4)	0.50
Lesion occurrence independent of UE	—	2 × 2 (rows 1–2 vs 3–4)	0.0098

*Note: UE and IA indicate the combined effect of UE and IA with four levels: 2.74 MPa and saline, 2.74 MPa and Optison®, 5.86 MPa and saline, and 5.86 MPa and Optison®.

The only significant effect that was different between the investigation by Raeman et al.[5] and our study was the size (surface area) of the lesion when the contrast agent was in the vascular system. They found that the surface area was about 19 mm^2 at an in situ peak rarefactional pressure of 1.4 MPa whereas ours was zero (no lesions) at an in situ peak rarefactional pressure of 2.74 MPa and 0.48 ± 0.25 mm^2 at an in situ peak rarefactional pressure of 5.86 MPa. We hypothesize that this difference is due to the large difference in exposure time, 300 seconds for theirs and 10 seconds for ours, because we did not find any differences between rats and mice in our exposure-effect study.[13]

Our results are also consistent with those of Dalecki et al.,[25] who used the same mouse strain and contrast agent as Raeman et al.,[5] but used a lithotripter field to expose the mice (Table I).

Further, to show that the results reported herein are consistent with our previous studies, lesion occurrence, depth, surface area, and volume from the four IA × UE data sets have been superimposed (Fig. 2) on our previous exposure-effect study[13] that evaluated the effects of species difference (mice vs rats) and

TABLE IV

Censored Rank Test Analysis of Lesion Size (Depth and Surface Area)

Null Hypothesis	Conditioning Variables	One-Sided P-Value
No IA effect on	UE = 2.74 MPa	0.50
lesion depth	UE = 5.86 MPa	0.41
No IA effect on	UE = 2.74 MPa	0.50
lesion surface area	UE = 5.86 MPa	0.48
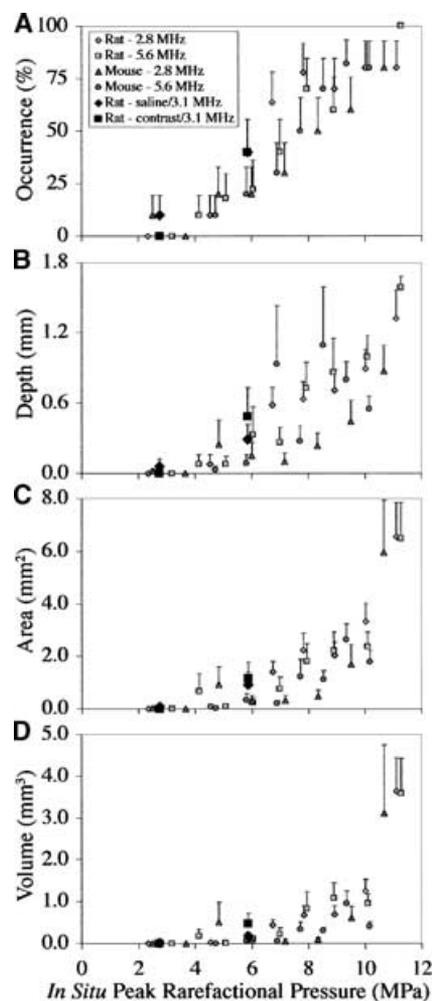
Figure 2. *Rat lung hemorrhage* **(A)** *occurrence,* **(B)** *depth,* **(C)** *surface area, and* **(D)** *volume as a function of the in situ peak rarefactional pressure that compare the four IA (saline and Optison®) and UE (in situ peak rarefactional pressures of 2.74 and 5.86 MPa) data reported herein (solid squares and diamonds) to the previously published mice and rats data.[13] Error bars are SEM.*

frequency (2.8 and 5.6 MHz). The comparison shows that they are essentially the same.

In summary, while it is not possible to prove a negative, what has been shown herein is that when Optison® is circulating in the vascular system of rats, lesion occurrence and size are essentially the same as when there is no contrast agent. Also, our results are in general agreement with two other studies[5,25] that showed similar results using a different species, a different contrast agent and different UE conditions. Although our negative hypothesis has not been proved, the accumulating evidence strongly suggests that inertial cavitation is not the physical mechanism responsible for ultrasound-induced lung damage.

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