Published in final edited form as:

Ultrasound Med Biol. 2009 September ; 35(9): 1574–1586. doi:10.1016/j.ultrasmedbio.2009.04.013.

An Ex Vivo Study of the Correlation between Acoustic Emission and Microvascular Damage

Stanley Samuel[†], Michol A. Cooper[†], Joseph L. Bull^{*}, J. Brian Fowlkes[†], and Douglas L. Miller[†]

[†]Department of Radiology, University of Michigan Medical Center, Ann Arbor, MI 48109 USA

^{*}Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109 USA

Abstract

The objective of this study was to conduct an *ex vivo* examination of correlation between acoustic emission and tissue damage. Intravital microscopy was employed in conjunction with ultrasound exposure in cremaster muscle of male Wistar rats. Definity® microbubbles were administered intravenously through the tail vein ($80 \mu\text{L} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ infusion rate) with the aid of a syringe pump. For the pulse repetition frequency (PRF) study, exposures were performed at four locations of the cremaster at a PRF of 1000, 500, 100 and 10 Hz (one location per PRF per rat). The 100-pulse exposures were implemented at a peak rarefactional pressure (P_r) of 2 MPa, frequency of 2.25 MHz with 46 cycle pulses. For the pressure amplitude threshold study, 100-pulse exposures (46 cycle pulses) were conducted at various peak rarefactional pressures from 0.5 MPa to 2 MPa at a frequency of 2.25 MHz and PRF of 100 Hz. Photomicrographs were captured before and 2-minutes post exposure. On a pulse-to-pulse basis, the 10 Hz acoustic emission was considerably higher and more sustained than those at other PRFs (1000, 500, and 100 Hz) ($p < 0.05$). Damage, measured as area of extravasation of red blood cells (RBC's), was also significantly higher at 10 Hz PRF than at 1000, 500, and 100 Hz ($p < 0.01$). The correlation of acoustic emission to tissue damage showed a trend of increasing damage with increasing cumulative function of the relative integrated power spectrum (CRIPS; $R^2 = 0.75$). No visible damage was present at $P_r \leq 0.85$ MPa. Damage, however, was observed at $P_r \geq 1.0$ MPa, and it increased with increasing acoustic pressure.

Keywords

ultrasound; bioeffects; tissue damage; contrast agent; acoustic emission; correlation

Introduction

Contrast agents for diagnostic ultrasound consist of a suspension of stabilized gas bubbles (i.e., gas bodies). These gas bodies act as cavitation nuclei in the presence of an appropriate acoustic field, which can result in a strong enhancement of acoustic emissions available for imaging applications (Forsberg and Shi 2001). Techniques employed to take advantage of such emissions include, but are not limited to, stimulated acoustic emission (loss of correlation imaging), and phase/pulse-inversion mode (PIM) (Harvey et al. 2001). However, many studies have shown that contrast-enhanced diagnostic ultrasound (US) can induce bioeffects both *in vitro* and *in vivo*, such as petechial hemorrhage, capillary damage, nephron injury, hemolysis and cell sonoporation (Miller et al. 2001; Skyba et al. 1998; Miller and Qudus 2000; Li et al.

2004; Williams et al. 2007; Miller 2007b). Inertial cavitation (IC) is believed to be the main cause for these nonthermal ultrasound bioeffects (Apfel and Holland 1991; Miller and Thomas 1995; NCRP 2002).

During previous *in vitro* studies involving monolayers, cells were forced into contact with contrast microbubbles prior to insonation in order to facilitate attachment. Thus the movement of the microbubbles was limited to the space between dual layers of the chamber window (Miller and Qudus 2000; Amararene et al. 2001). The presence (or disappearance) of bubble signal was presented as an indication of cavitation activity adequate for cellular bioeffects at high contrast concentrations (Samuel et al. 2006). These effects *in vitro* were examined pulse-to-pulse at each exposure, monitored through cell death, for correlation to acoustic emissions concomitant with optical feedback (Samuel et al. 2009).

Important questions remain as to the behavior of microbubbles *in vivo* and, by extension, their effect on tissue in a clinical setting. It is known that intravascular inertial cavitation activity can be induced *in vivo* with ultrasound for which the microbubbles serve as nuclei. A dose-responsive manner of cavitation activity occurs with respect to peak rarefactional pressure, ultrasound frequency, pulse repetition frequency (PRF), and pulse duration (Everbach et al. 1997). Such cavitation activity can cause rupture of the microvessel in which it occurs, with red blood cell (RBC) extravasation into the nearby interstitial space (Song et al. 2002; Maruvada and Hynynen 2004; Miller et al. 2007a). Studies have also been conducted to detect and quantify inertial cavitation activity *in vivo* (Tu et al. 2006b) and to correlate it with endothelial cell damage (Hwang et al. 2006). However, vascular damage was examined by excising the tissue, pressure-fixing it and employing light or electron microscopy. There was no direct real-time visualization of damage. Therefore, tests developed to address this lack employed intravital microscopy for real-time optical feedback. The influence of PRF on tissue damage and the pressure amplitude threshold for tissue damage were examined. Acoustic exposure of contrast microbubbles within the vasculature of a live animal, complemented by real-time optical feedback, could further the understanding related to effects *in vivo*, whether unwanted (bioeffects) or desired (drug and gene delivery).

Methods

Animal Preparation

All *ex vivo* animal procedures in this study were conducted with the approval and guidance of the University Committee on Use and Care of Animals at the University of Michigan. A schematic diagram for intravital microscopy of the rat cremaster muscle during acoustic exposure is shown in Fig. 1. The cremaster is the muscular pouch surrounding the testis of the male rat. External stimuli such as temperature changes induce it to raise the testis into and lower it from the abdomen. The procedure used for the cremaster muscle preparation was adapted from previous protocols for open muscle cremaster preparation for intravital microscopy studies (Baez 1973). Male rats of the Wistar strain procured from Charles River Laboratories (Wilmington, Massachusetts, U.S.A) weighing between 150 and 300 g were used. Anesthesia was performed using 75 mg/kg ketamine (Fort Dodge Animal Health, Iowa, U.S.A.) and 20 mg/kg xylazine (Fort Dodge Animal Health, IA, U.S.A.) administered through an IP injection. A BD Angiocath 26 gauge catheter (BD Medical Systems, Sandy, Utah, U.S.A.) was inserted into the tail vein for microbubble injection. The anterior aspect of the scrotum was then gently shaved and loose hairs were removed. With the rat in the supine position, a longitudinal incision of skin and fascia were made in the midline over the ventral aspect of the scrotum extending from 5-8 mm above inguinal fold to the distal end of the scrotum. The cremaster sack was gently excised using forceps, and the connective tissue fascia was carefully separated by blunt dissection around the cremaster sack. A 5 mm lateral incision was made at the top of the cremaster muscle taking care to avoid the larger anastomosing vessels. The

epididymus and testis was extracted through the incision and the cremaster pouch was turned inside out. The cremaster artery and vein that attach the testis and the cremaster pouch remained intact. This inversion procedure to expose the cremaster muscle was significantly less invasive than previous protocols, where a cut was made along the edge of the entire cremaster (7 - 9 cm in length), after which, the cremaster was splayed for viewing. The inside-out cremaster pouch was stretched out radially by hand. There were two layers of the muscle because the cremaster pouch was kept intact and not splayed. 5-0 Dexon "S" sutures (Davis-Geck, Manati, Puerto Rico, U.S.A.) were placed along the edges of the muscle at 1 cm increments, leaving 6 cm of suture at each location to aid in stretching the tissue over the viewing area. Throughout the procedure, a 10 cm × 10 cm gauze (Kendall, Mansfield, MA, U.S.A.) soaked with 37 °C saline was placed on the cremaster to prevent the tissue from dehydrating and to maintain the appropriate temperature at the tissue location. This ensured minimal compromise of the blood flow due to extraction of the cremaster muscle. Intravital microscopy comparison of the tissue with, and without, the wet gauze showed a marked difference in the capillary blood flow. Tissue preparation without the use of wet gauze resulted in sluggish or halted blood flow whereas that with wet gauze (37°C) showed normal blood flow. Tape was used to secure the ends of the suture at each suture location.

The rat was then placed in the prone position on a ramp in a Plexiglas tank to allow its head to be above water during experiments. A 3.2 cm diameter hole centered in the bottom of the tank provided an optical window. A 10 cm × 10 cm square of polycarbonate clear film (0.125 mm thickness) was placed over the hole and sealed in place with duct tape. Duct tape, among other tapes and adhesives that were employed, provided the best combination of being leak proof during the course of the experiment and disposable following each experiment. The 0.125 mm polycarbonate film was used in the optical window due to the short focal lengths of the high power microscope objectives and to its ease of disposal following each experiment.

Standing waves, potentially caused by the ultrasound beam being reflected back to the same location in the tissue, could create an unknown increase or decrease in the exposure. A circular aluminum platform, supported on 4 legs, was fabricated in order to raise the tissue from the bottom of the tank and minimize standing waves (see Fig. 2). The platform height was 11 mm. The inner diameter of the platform's circular window was 34.3 mm. The platform was centered on the optical window at the bottom of the tank. The legs of the platform were anchored to the bottom of the tank using J-B Kwik glue (J-B Weld, Sulphur Springs, Texas, USA). A piece of polycarbonate film (5 cm × 5 cm square) served as the tissue holder on the platform. The cremaster was radially stretched and attached to the holder through the use of sutures. The film was optically transparent. Acoustic properties of the film include an intensity reflection coefficient of 0.02 (water-polycarbonate interface, Onda Corporation, Sunnyvale, California, USA) and attenuation of 0.31 dB at 5 MHz (for 0.127 mm thick film) (Onda 2003). These favorable optical and acoustic properties of the film allowed its use as the material of choice for a tissue holder. The testis was kept intact and placed adjacent to the platform in order to facilitate normal blood flow to the cremaster vasculature and to ensure that the acoustics and optics were unimpeded. The tank was filled with 37 °C degassed saline and placed on the platform of the Leica inverted microscope (model DMIL, Bannockburn, Illinois, U.S.A.).

Optics

A Mitutoyo 5× long-working-distance (LWD) objective (Model M Plan Apo 5×, Mitutoyo America Corp., Aurora, IL, USA) was employed for optical viewing of the tissue target. Video clips were captured during exposures using a CoolSNAP camera (Model ES, Photometrics, Tucson, Arizona, USA) connected to the inverted microscope. Pre-exposure and post-exposure photomicrographs were also captured using the same setup. The camera has a monochrome cooled 2/3" CCD providing 12-bit digitization at 20 MHz. A 1392×1040 imaging array and

6.45×6.45µm pixels make it ideally suited to optical microscopes. The camera was controlled via software provided by the vendor (Metamorph, Photometrics, Tucson, AZ, USA). Power and data were transferred through a low voltage differential signaling (LVDS) 20-pin high-density connector that required a specific data acquisition board, also provided by the vendor. The tissue was illuminated using direct light from a 150W metal halide light source (Techniquip, Pleasanton, CA, USA). The light intensity provided by such a source was sufficient for capturing video clips at 30 frames per second (fps). Owing to limitations in the trigger options for the camera, the total time for capture of video frames was set at 5 seconds for the 100, 500, and 1000 Hz PRFs and 15 seconds for the 10 Hz PRF. Since the camera software did not accommodate an external trigger feature, it was triggered separately ~1 second before the acoustics and the data acquisition system. The optical and acoustical data acquisition systems were, therefore, not completely synchronous.

Acoustics

The setup for the acoustical component of this study was similar to previous studies (Samuel, et al 2006). A programmable function generator (Model 3314A, Hewlett Packard, Palo Alto, CA, USA) produced sinusoid pulses containing 46 cycles (pulse duration of 20.4 µs) at a center frequency of 2.25 MHz. Long pulses (>32 cycles) have been employed in the past to achieve sufficient signal-to-noise ratio or narrow bandwidth for measurement of weak signal components such as the subharmonic and ultraharmonic components in spectra of scattered signals (Shi and Forsberg 2000). The use of 46 cycles was both to account for the transient response in the received signal and to ensure good signal-to-noise ratio. The 1 MHz receive transducer used in all the studies underwent a ring-up to resonance and a ring-down since the ultrasound signals were generated using a 2.25 MHz transducer (off-resonance frequency). Both the ring-up and ring-down covered 5 cycles each, requiring the received signal to be range gated for the central portion leaving 35 cycles in order to ensure good signal to noise ratio. The HP3314A was triggered by another function generator (Model 33120A, Hewlett Packard, Palo Alto, CA, USA), which generated square pulses of 100 cycles each and PRFs of 1000Hz, 500 Hz, 100 Hz, and 10 Hz in order to maintain consistent acoustical parameters while transitioning from an *in vitro* model (Samuel et al. 2006; Samuel et al. 2009) to an *in vivo* one. Each cycle of the HP 33120A corresponded to a pulse generated by the HP 3314A. A 55-dB RF power amplifier (ENI Model A-300, MKS Instruments, Andover, MA, USA) was used for amplifying the output from the function generator. The amplified signal was supplied to a 2.25 MHz transmit transducer (A305S, Panametrics, Waltham, MA, USA). Except for the threshold study, each exposure was at a P_r of 2 MPa in degassed saline at 37°C. For the threshold study, exposures were conducted at peak rarefactional pressures of 0.5, 0.75, 0.85, 1.0, 1.5, and 2.0 MPa. Table 1 below provides a summary of the exposures for the threshold study. The scattered signals from the bubbles/tissue were detected using a 1 MHz transducer (A314S, Panametrics, Waltham, MA, USA) due to its sensitivity to the subharmonic response of the bubbles. The transducers were placed in perpendicular planes to each other, and at an approximate angle of 45° to the plane of the tissue in order to limit the reception to merely the incoherent component of scattering from the tissue. They were aligned confocally on the tissue. Each transducer was 19 mm in diameter and spherically focused at 38 mm. The lateral beam width at the focus of each transducer was 2.3 mm (-6 dB FWHM). The -6dB bandwidth of the transmit transducer was 74% while that of the receive transducer was 66%. Both the transmit and the receive transducers were calibrated using a planar hydrophone (Reference Shock Wave Hydrophone™, Sonic Industries, Hatboro, Pennsylvania, USA). The field of view of each video frame was 1800µm × 1350µm as measured through the use of a stage micrometer (10 microns/div). The received signals were amplified by 50 dB (Model MR 106 receiver, MetroTek, Beaverton, Oregon, USA) before being digitized.

Data Acquisition Equipment and Processing Algorithms

The efficacy of acoustic emissions as a predictor of bioeffects or therapy was to be investigated through its correlation with optical images. Similar to a previously used approach, a high speed digitizing board (PDA12A-125 MHz, Signatec, Corona, CA, USA) in conjunction with LabVIEW (National Instruments, Austin, TX, USA) was used in the acquisition of the scattered signal $s_p(t)$ from the microbubbles and tissue as sensed by the receive transducer, where p is the pulse number (Samuel, et al 2006). For the sole purpose of this study, “sham exposure” is defined as ultrasound exposure of the tissue employing the same pulsing parameters as regular exposure but in the absence of contrast. One reference measurement (100 pulses), provided by sham exposure, was obtained for each of the rats at each location where subsequent exposure was performed with contrast. Each reference measurement is given by $r_p(t)$, where p is pulse number. The data set for each exposure was transferred to a computer for analysis using MATLAB (MathWorks, Inc., Natick, MA, USA). A Hamming window of 16 μ s duration (36 cycles for a 2.25 MHz pulse) for the 46 cycle exposures was used to range gate for the central portion of the received pulse from the tissue. The frequency spectrum for each pulse, $s_p(f)$ was obtained using Fast Fourier Transform (FFT) on the windowed signal. Range gating employing 36 cycles ensured good signal-to-noise ratio and exclusion of the transient response of the receive transducer (ring up and ring down) (Shi and Forsberg 2000). The power spectrum $s_p^2(f)$ was calculated. The reference power spectrum, $r^2(f)$ was calculated for each corresponding location of the contrast exposure by averaging the power spectrum of the scattered signal across 100 pulses. The Relative Integrated Power Spectrum (RIPS) was, then, computed as

$$\text{RIPS}_n = \int_{f_1}^{f_2} \frac{s_p^2(f)}{r^2(f)} df \quad \text{where } n \text{ designates the rat on which the exposure was performed.} \quad (1)$$

The power spectrum, $s_p^2(f)$, is normalized with the reference power spectrum, $r^2(f)$. Total RIPS is calculated by adding the subharmonic RIPS and the first ultraharmonic RIPS. Subharmonic RIPS is a summation of the normalized power spectrum across a frequency bandwidth ($f_2 - f_1$) of 1 MHz, centered on the subharmonic ($1/2f_0 = 1.125$ MHz). The first ultraharmonic RIPS is a similar summation but centered on the first ultraharmonic ($3/2f_0 = 3.375$ MHz). RIPS provides a comparison of acoustic power of scattering from active microbubbles in tissue with that from tissue alone.

Quantification of Tissue Damage

Optical examination was used to establish endpoints for both the PRF study and the threshold study through assessment of the exposure region for capillary leakage into the interstitium. This was accomplished by capturing pre- and post-exposure photomicrographs for each exposure. Post-exposure images were captured 2 minutes following exposure to allow enough time, if needed, for adjustment of equipment settings. An automated approach and an operator-based approach were compared. The bioeffect observed was extravasation of blood into the observed tissue from damaged blood vessels. Owing to limitations of the intravital microscopy preparation, a simple measure of the fraction of the observed area opacified by extravasated blood was developed. This measure could not be directly related to post-exposure methods, such as measurement of microvascular leakage of dye or counts of petechial hemorrhages, which have been used, for example, in rat heart (Li et al. 2004).

For the automated approach, both the pre- and post-exposure images were first self-normalized to go from 0 to 1 (floating point precision). This allowed thresholds to be set for the subtraction

images (pre minus post) between 0 and 1. Histogram equalization was applied to each of the normalized images in order to account for differing background intensities between the pre- and post-exposure set. Different background intensity in a region between the pre- and post-exposure image could result in a larger difference value in that region following subtraction, which could lead to an erroneous decision on extravasation. Histogram equalization takes an image's accumulated normalized histogram, equivalent to the cumulative distribution function, and linearizes it across a value range, in this case 0-255. Subtraction was performed between pre-exposure and post-exposure image following histogram equalization. Pixels representing dark regions (extravasation regions) were more positive after subtraction. A binary mask was created using a threshold value between 0 and 1. Every white pixel in the binary mask represented extravasation and every black pixel was background tissue and vasculature common to both images (Figs. 3c and 3g). The over or underestimation of damage was controlled by the threshold value (higher values tended to over-estimate the damage). A threshold value providing the best approximation of the region of extravasation was selected through trial and error. The value applied for this study was 0.16. Once the appropriate value was selected for one pre-exposure and post-exposure set, the same value was applied to all sets of images. The number of white pixels (extravasation) in the resulting mask was divided by the total number of pixels in the image to provide the percent area of extravasation. The mask was superimposed on to the post-exposure image for better visualization of the regions counted as "darker" pixels when compared with the pre-exposure image (Figs. 3d and 3h).

The automated approach to quantifying extravasation was compared with that employing an operator in order to validate the automated method. Images captured at a particular magnification were superimposed with micrometer images (both horizontal and vertical alignment of the micrometer) at the same magnification using the *Merge Layer* feature of Photoshop (Version CS2, Adobe Systems Inc., San Jose, CA, USA). A grid was, then, drawn on the image using the graduations of the micrometer. Each square on the grid represented $50\mu\text{m}^2$. The number of squares (including fractions) overlapping the area of hemorrhage was tabulated for quantification of the hemorrhagic area (Figs. 3a, 3b, 3e, and 3f).

Experimental Design

Two main studies were conducted: (i) the effect of PRF on tissue damage and, (ii) the pressure amplitude threshold for tissue damage. Five rats were used in the first study and three in the second. Sham exposures, detailed below, were performed on each rat before, and after, contrast was administered. Immediately following the first type of sham exposures, 0.3 mL of contrast (Definity®, Bristol-Myers Squibb Medical Imaging, N. Billerica, MA, USA) was diluted 10× in saline. Definity® was used for this study owing to its greater robustness in the vasculature and lower sensitivity to acoustic pressure amplitudes when compared with Optison® (Moran, et al. 2000). The resulting suspension was administered through the cannulated tail vein with the aid of a syringe pump driving at a volume flow rate of 0.24 mL per minute ($80\mu\text{L.kg}^{-1}.\text{min}^{-1}$ infusion rate). In comparison, currently approved clinical dosage in humans for continuous intravenous infusion is $1\text{--}3\mu\text{L.kg}^{-1}.\text{min}^{-1}$ (definityimaging.com). Rats were weighed after anesthesia to determine appropriate contrast administration. Definity® has a bubble concentration of 1×10^9 bubbles/mL with a size range of 1-4 μm diameter. Each exposure was performed following contrast administration for 1 minute. The syringe pump continued to operate during the exposure but was paused immediately following each exposure to allow for re-suspension of the bubbles that had risen to the top of the syringe chamber. Each post-exposure photomicrograph was taken after a 2-minute delay.

PRF Study

Two types of sham exposures were carried out for each rat and at each location. The first type was used for calculation of the reference power spectrum and RIPS mentioned earlier and the

second type was used for comparing tissue damage. For the first type of sham, for each rat, exposures were performed at four unique locations of the cremaster, each at one of the four PRFs being investigated (1000, 500, 100, and 10 Hz). The cremaster of one of the animals was sufficiently larger than the others so as to allow an additional exposure. 500 Hz PRF was chosen arbitrarily for exposure at the fifth location for this rat. The locations for sham exposure were chosen in such a way as to ensure clear observation of the vasculature and blood flow at each location. The second type of sham exposures involved turning off the acoustics from the first sham and administering the contrast agent. Video was captured at each of the previously mentioned locations to investigate damage from the mere presence of contrast microbubbles in the vasculature. Once all the data associated with sham were collected, exposures were performed at each of the four PRFs being investigated, one exposure per PRF. The exposures were performed at the same location as the sham (four locations total per rat). The order of the PRFs was randomized between rats in order to reduce any systematic error. Exposures were performed employing the pulsing parameters mentioned above. We decided arbitrarily to use the 500Hz PRF. Real-time video and scattered signals were captured simultaneously using the arrangement described under the *Optics* and *Acoustics* sections.

Pressure Amplitude Threshold Study

Sham exposures were performed at five different locations for each rat, corresponding to the peak rarefactional pressures at which exposures were to be performed following the administration of contrast. Table 1 below summarizes the number of exposures that were carried out per peak rarefactional pressure.

Results

Figures 3a and 3e show images of the rat cremaster muscle before exposure of the region to ultrasound. Figures 3b and 3f show the same respective regions after exposure to ultrasound at a frequency of 2.25 MHz, 2 MPa of peak rarefactional pressure, 100 pulses at 46 cycles per pulse. PRFs of 1000 Hz (Fig. 3b) and 10 Hz (Fig. 3f) are compared. A grid was superimposed on each image to allow for quantification of the area of extravasation. Areas of extravasation are identified by dashed outlines in Figs. 3b and 3f. Binary masks produced by the automated approach are shown in Fig. 3c and 3g. Each white pixel on the mask represents extravasation. Figures 3d and 3h are images where the binary mask was superimposed on the post-exposure image. This allowed better visualization of the regions counted as “darker” pixels when compared with the pre-exposure image. Each square on the grid is $50\mu\text{m}^2$. The field of view is $1800\mu\text{m} \times 1350\mu\text{m}$. These images are representative and show considerably greater damage at the 10 Hz PRF (Figs. 3f and 3h) than at 1000 Hz (Figs. 3b and 3d). There was also good agreement between the areas of damage as assessed by the operator (Figs. 3b and 3f) and the automated approach (Figs. 3d and 3h). This agreement is corroborated in Fig. 5.

Figure 4(a) presents the acoustic emission, for all pulses, as the median of the Relative Integrated Power Spectrum (RIPS) of the microbubble activity within the region of the rat cremaster exposed to ultrasound. Median was chosen as the indicator of central tendency as the population sample was small ($n=5$) and some values were prone to extremes which would have resulted in a skewing of the arithmetic mean. The vertical bars indicate the range of values in the population sample. The RIPS is a combination of the subharmonic and the first ultraharmonic response. A log scale representation of pulse number clearly shows the acoustic response during the first 20 pulses. The RIPS associated with the 100 Hz and 500 Hz PRFs behave similarly, reducing monotonically to noise by the sixth pulse. The emission at the 1000 Hz PRF is sustained till the ninth pulse before reducing to noise by the twentieth pulse. For the 10 Hz PRF, however, the emission is greater (~ 2 times that of the maximum at other PRFs) and more sustained. There is a reduction in the acoustic activity by the fiftieth pulse during the

10 Hz regime but it is only to a level of maximum scattering seen during exposures involving the other PRFs (100, 500, and 1000 Hz). The RIPS is, then, sustained at that level for the duration of the exposure (100 pulses).

Each data point is plotted with a minimum-maximum range to assess if acoustic responses at the different PRFs are statistically different. On a pulse-to-pulse basis, the 10 Hz response is statistically different from those at other PRFs (1000, 500, and 100 Hz) (Mann-Whitney, directional test, $n_A=n_B=5$, $p < 0.05$). The 1000 Hz response is shown to be sustained for a longer period, and is statistically different for pulses three through twenty (Mann-Whitney, directional, $p = 0.05$), than the 500 or 100 Hz responses. For all other pulses, the response from 1000, 500, and 100 Hz PRFs is statistically similar. The first pulse produces similar responses at all PRFs. This is anticipated because the changes in PRF, which may affect bubble evolution, are not experienced by the bubbles during the first pulse.

Figure 4(b) displays the RIPS from exposures at 1000, 500, 100, and 10 Hz PRFs as a function of time. Various PRFs correspond to different time intervals at which the microbubbles are insonified. The purpose of this plot was to identify the contribution of time, if any, in the evolution of the microbubbles and their response to acoustic pulses. Each data point corresponds to a pulse at the particular PRF; therefore, only two pulse responses are shown for the 10 Hz PRF, while the 1000 Hz PRF shows hundred data points. The 10 Hz response is, again, considerably higher than that at the other PRFs (Mann-Whitney directional, $p < 0.05$). Although not statistically different, there is a trend lower from 100 Hz to 500 or 1000 Hz. The 10 Hz response continues to rise at 100 ms, the 100 Hz and 1000 Hz responses reduce to noise by 20 ms, while the 500 Hz response requires only 10 ms to reach the noise floor.

Tissue damage within the vasculature of the rat cremaster due to the insonification of microbubbles is presented as an area of extravasation in Fig. 5. The area of extravasation, calculated as a percentage of the total area in the field of view, is shown for various PRFs. Values are expressed as mean with vertical bars representing one standard deviation. Damage was 15% ($\pm 12\%$ SD) at 1000 Hz PRF, 22% ($\pm 11\%$ SD) at 500 Hz, 21% ($\pm 9\%$ SD) at 100 Hz, and 55% ($\pm 13\%$ SD) at 10 Hz. Unlike the RIPS values, these data do not deviate significantly from the normal to require transformation before application of the t-test. Damage for 1000, 500, and 100 Hz PRFs is statistically similar, whereas, it is significantly higher for the 10 Hz PRF (t-test, $p < 0.01$). No microvessel ruptures were seen after ultrasound exposure before infusion of microbubbles (sham type 1). Similarly, no ruptures were seen in the absence of ultrasound when microbubbles were infused (sham type 2). These sham readings are included in Fig. 5. Analysis of the results comparing damage assessed by the automated approach (open markers) and that by the operator (closed markers) shows that the two approaches are statistically equivalent.

The correlation of tissue damage to acoustic emission was examined. Any effect on the tissue would have been a cumulative one from all the pulses since damage was only measured at the end of each exposure. As a result, the cumulative function of the RIPS (CRIPS), defined earlier, was used as the emission indicator (Amararene, et al. 2001). Briefly, CRIPS was calculated by the summation of RIPS from each pulse (100 pulses). The percent area of extravasation, as a function of CRIPS, was plotted as a scatter plot (Fig. 6). A least squares fit to this plot shows a trend of increasing damage with increasing CRIPS. The trendline has a correlation coefficient, $R^2 = 0.75$. Dashed lines in the figure split the extravasation-emission relationship into four quadrants. The dashed lines were chosen as the mid-point of damage values (horizontal line) and an order of magnitude above and below the min and max CRIPS values, respectively. The largest extravasation and emission is produced at the 10 Hz PRF. Data points representing exposures at 10 Hz PRF mostly occupy the upper right quadrant. In contrast, all exposures at

the higher PRFs occupy the lower left quadrant indicating lower emissions and associated extravasation.

The peak rarefactional pressure threshold for damage was also investigated at 100 Hz PRF. Figure 7 shows a scatter plot of the tissue damage (percent area of extravasation) as a function of the peak rarefactional pressure (P_r). No visible damage was present at $P_r \leq 0.85$ MPa as the plot depicts. Damage, however, is observed at $P_r \geq 1.0$ MPa and it increases with increasing acoustic pressure. The least squares fit of the increasing trend, employing nine data points, shows a clear correlation ($R^2 = 0.98$). Each data point represents an exposure at a particular peak rarefactional pressure. There were two data points at 0.85 MPa which were used for the nine-point regression line. Data below 0.85 MPa were excluded from the regression as this would have skewed the low end of the line towards the lower pressure amplitudes thereby indicating damage at 0.85 MPa where there was none.

Discussion

Acoustic Emission

In vitro studies have shown that the presence of ultrasound contrast agents can increase IC activity, which, in turn, can be monitored via IC “dose” (ICD). ICD is defined as the cumulated r.m.s broadband noise amplitude in the 3.85 MHz (± 0.2) (Chen et al. 2003) or 20 MHz (± 1) (Everbach et al. 1997) band in the frequency domain. The term “acoustic emission” (quantified as RIPS) is used throughout this text instead of IC dose due to the detection of inertial cavitation using a slightly different technique than those presented by Everbach et al. The results of a previous *ex vivo* study (Tu et al. 2006a) are consistent with the results of this study where the acoustic response to the 10 Hz PRF is greater and more sustained than the higher PRFs (100, 500, 1000 Hz). The reason for this effect is probably the replenishment of microbubbles in the insonation zone.

The capillary flow velocity in rat cremaster muscle is 200 - 300 $\mu\text{m}/\text{sec}$ (Braide et al. 2006; Lombard et al. 2000). Both centerline RBC velocity and mean microbubble velocity are well correlated for both arterioles and venules (Lindner, et al. 2002). Given the rat body weight (BW), in this case $\sim 300\text{g}$, its blood volume (ml) can be ascertained using the formula $0.06 \times \text{BW} + 0.77$, yielding 18.77 mL or 62.57 mL/kg (normalized to its body weight) (Lee and Blafox 1985). The contrast dosage based on this calculation is 27 μL per mL of blood. Due to a $10\times$ dilution of the contrast before injection, the number of microbubbles would have been 1×10^8 per mL of infusion solution. Assuming a median microvascular diameter of 20 μm based on previous empirical results (Lindner, et al. 2002), any given point on each vessel in the field of view would theoretically see 2 or 3 microbubbles passing every 10 seconds. The likelihood of these bubbles flowing through each vessel synchronously in the insonation zone is very low. However, it is highly probable that each pulse, spaced apart by 100 ms at 10 Hz PRF, would find some microbubbles in its focus. Therefore, the major component of the acoustic emission at 10 Hz PRF would probably come from the response of the microbubbles replenishing the insonation zone after each pulse.

However, at higher PRFs (≥ 100 Hz) the acoustic emission seems to be governed by slightly different bubble distributions. Based on the capillary flow velocity of 200 – 300 $\mu\text{m}/\text{sec}$ in rat cremaster, it would take ~ 5 seconds for an intact microbubble to traverse the field of view. At PRFs of 1000, 500, and 100 Hz, the total exposure time is ≤ 1 second, which does not seem to provide sufficient time for replenishment of the zone with previously unexposed microbubbles. Under this regime, the main contribution to the acoustic response probably comes from the same microbubbles insonated by subsequent pulses until they are no longer resonant or have undergone dissolution. These mechanisms have been explored previously (Samuel et al. 2009).

Microvascular Damage

The dichotomy in acoustic response between the $\text{PRF} \leq 10$ Hz and $\text{PRF} \geq 100$ Hz is reflected in the corresponding microvascular damage. The results of damage from this study show that there is significantly greater damage at 10 Hz PRF than the other PRFs ($p < 0.01$). The results at higher PRFs are consistent with those in the previous *in vitro* studies (Samuel, et al. 2006; Samuel, et al. 2009) where no change in damage was observed beyond 100 ms of total exposure duration.

Petechial hemorrhages can result from extravasation points created due to inertial cavitation of contrast microbubbles. Replenishment of the bubbles within the field of view following each pulse would provide additional vehicles for the creation of extravasation points or enlargement of previously generated extravasation points at 10 Hz PRF. The cumulative effect over 100 pulses (as employed for this study) would result in significantly greater damage within the insonation zone. However, given the concentration of bubbles present in the blood volume and the capillary flow velocities related to this study, the total exposure time at higher PRFs (100, 500, and 1000 Hz) does not seem sufficient for microbubble replenishment. Therefore, the exposure area, regardless of PRF, would have similar number of cavitation nuclei available for the creation of extravasation points resulting in similar levels of microvascular damage. Note that petechial hemorrhages were not quantified in this study.

Correlation of Emission with Microvascular Damage

The study of the extravasation-emission relationship reveals that cumulative function of the RIPS (CRIPS) may find use as a determinant of microvascular damage due to contrast mediated ultrasound *in vivo*. There appear to be two regimes of acoustic exposure based on PRF, as indicated by the quadrants in Fig. 6. This raises the prospect that acoustic emission can be used as a possible predictor of ultrasound-induced bioeffects with the development of a model with a “replenishment PRF”, so that subsequent acoustic pulses insonify an equivalent volume of contrast agent. The upper right quadrant may be referred to as the “replenishment region” and the lower left quadrant, the “dissolution region.” In therapeutic applications, such as drug delivery, the extent of desired extravasation would dictate the choice of region used. Even though the scatter plot of the area of extravasation to the cumulative RIPS (CRIPS) shows a notable trend (Fig. 6), exposures at PRFs between 100 Hz and 10 Hz need to be examined in order to understand the transition between the two zones.

Acknowledgments

The authors thank Kimberly Ives and Chunyan Dou for assistance with animal preparation. This study was supported by grants EB00338 and DK 42290 awarded by the National Institutes of Health.

References

- Amararene A, Fowlkes JB, Miller DL. Relationships between scattered signals from ultrasonically activated contrast agents and cell membrane damage *in vitro*. Proc IEEE Ultrason Symp 2001;2:1751–1754.
- Apfel RE, Holland CK. Gauging the likelihood of cavitation from short-pulse, low-duty cycle diagnostic ultrasound. Ultrasound Med Biol 1991;17:179–185. [PubMed: 2053214]
- Baez S. An open cremaster muscle preparation for the study of blood vessels by *in vivo* microscopy. Microvasc Research 1973;5:384–394.
- Braide M, Rasmussen H, Albrektsson A, Bagge U. Microvascular behavior and effects of sonazoid microbubbles in the cremaster muscle of rats after local administration. J Ultrasound Med 2006;25:883–890. [PubMed: 16798899]
- Chen W, Brayman AA, Matula TJ, Crum LA. Inertial cavitation dose and hemolysis produced *in vitro* with or without Optison. Ultrasound Med Biol 2003;29:725–737. [PubMed: 12754072]

- Everbach EC, Makin IRS, Azadniv M, Meltzer RS. Correlation of ultrasound-induced hemolysis with cavitation detector output *in vitro*. *Ultrasound Med Biol* 1997;23:619–624. [PubMed: 9232771]
- Forsberg, F.; Shi, WT. Physics of contrast microbubbles. In: Goldberg, BB.; Raichlen, JS.; Forsberg, F., editors. *Ultrasound Contrast Agents: Basic principles and clinical applications*. Vol. 2nd. London: Martin Dunitz; 2001. p. 15-24.
- Harvey, CJ.; Blomley, MJK.; Cosgrove, DO. Acoustic emission imaging. In: Goldberg, BB.; Raichlen, JS.; Forsberg, F., editors. *Ultrasound Contrast Agents: Basic principles and clinical applications*. Vol. 2nd. London: Martin Dunitz; 2001. p. 71-80.
- Hwang JH, Tu J, Brayman AA, Matula TJ, Crum LA. Correlation between inertial cavitation dose and endothelial cell damage *in vivo*. *Ultrasound Med Biol* 2006;32:1611–1619. [PubMed: 17045882]
- Lee HB, Blaufox MD. Blood volume in the rat. *J Nuc Med* 1985;26:72–76.
- Li P, Armstrong WF, Miller DL. Impact of myocardial contrast echocardiography on vascular permeability: comparison of three different contrast agents. *Ultrasound Med Biol* 2004;30:83–91. [PubMed: 14962612]
- Lindner JR, Song J, Jayaweera AR, Sklenar J, Kaul S. Microvascular rheology of Definity microbubbles after intra-arterial and intravenous administration. *J Am Soc Echocard* 2002;15:396–403.
- Lombard J, Frisbee J, Greene A, Hudetz A, Roman R, Tonellato P. Microvascular flow and tissue PO₂ in skeletal muscle of chronic reduced renal mass hypertensive rats. *Am J Phys – Heart Circ Phys* 2000;279:H2295–H2302.
- Maruvada S, Hynynen K. Optical monitoring of ultrasound-induced bioeffects in glass catfish. *Ultrasound Med Biol* 2004;30:67–74. [PubMed: 14962610]
- Miller DL, Thomas RM. Ultrasound contrast agents nucleate inertial cavitation *in vitro*. *Ultrasound Med Biol* 1995;21:1059–1065. [PubMed: 8553500]
- Miller DL, Quddus J. Sonoporation of monolayer cells by diagnostic ultrasound activation of contrast-agent gas bodies. *Ultrasound Med Biol* 2000;26:661–667. [PubMed: 10856630]
- Miller DL, Dou C, Wiggins RC, Wharram BL, Goyal M, Williams AR. An *in vivo* rat model simulating imaging of human kidney by diagnostic ultrasound with gas-body contrast agent. *Ultrasound Med Biol* 2007a;33:129–135. [PubMed: 17189055]
- Miller DL. WFUMB safety symposium on echo-contrast agents: *in vitro* effects. *Ultrasound Med Biol* 2007b;33:197–204. [PubMed: 17223252]
- Miller MW, Everbach EC, Cox C, Knapp RR, Brayman AA, Sherman TA. A comparison of the hemolytic potential of Optison and Albunex in whole human blood *in vitro*: acoustic pressure, ultrasound frequency, donor and passive cavitation detection considerations. *Ultrasound Med Biol* 2001;27:709–721. [PubMed: 11397535]
- Moran CM, Anderson T, Pye SD, Sboros V, McDicken WN. Quantification of microbubble destruction of three fluorocarbon-filled ultrasonic contrast agents. *Ultrasound Med Biol* 2000;26:629–639. [PubMed: 10856626]
- NCRP. Exposure criteria for medical diagnostic ultrasound: II. Criteria based on all known mechanisms. Report No. 140. Bethesda, MD: National Council on Radiation Protection and Measurements; 2002.
- Onda. Tables of acoustic properties of materials. 2003. www.ondacorp.com
- Samuel S, Miller DL, Fowlkes JB. The relationship of acoustic emission and pulse repetition frequency in the detection of gas body stability and cell death. *Ultrasound Med Biol* 2006;32:439–447. [PubMed: 16530103]
- Samuel S, Fowlkes JB, Miller DL. An *in vitro* study of the correlation between bubble distribution, acoustic emission, and cell damage by contrast ultrasound. *IEEE Trans Ultrason Ferro Freq Contr* 2009;56:589–599.
- Shi WT, Forsberg F. Ultrasonic characterization of the nonlinear properties of contrast microbubbles. *Ultrasound Med Biol* 2000;26:93–104. [PubMed: 10687797]
- Song J, Chappell JC, Qi M, VanGieson EJ, Kaul S, Price RJ. Influence of injection site, microvascular pressure and ultrasound variables on microbubble-mediated delivery of microspheres to muscle. *J Am Coll Cardiol* 2002;39:726–731. [PubMed: 11849875]
- Skyba DM, Price RJ, Linka AZ, Skalak TC, Kaul S. Direct *in vivo* visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* 1998;98:290–293. [PubMed: 9711932]

- Tu J, Matula TJ, Brayman AA, Crum LA. Inertial cavitation dose produced in ex vivo rabbit ear arteries with Optison[®] by 1-MHz pulsed ultrasound. *Ultrasound Med Biol* 2006a;32:281–288. [PubMed: 16464673]
- Tu J, Hwang JH, Matula TJ, Brayman AA, Crum LA. Intravascular inertial cavitation activity detection and quantification *in vivo* with Optison[®]. *Ultrasound Med Biol* 2006b;32:1601–1609. [PubMed: 17045881]
- Williams AR, Wiggins RC, Wharram BL, Goyal M, Dou C, Johnson KJ, Miller DL. Nephron injury induced by diagnostic ultrasound imaging at high mechanical index with gas body contrast agent. *Ultrasound Med Biol* 2007;33:1336–1344. [PubMed: 17507144]

1. A representative setup for intravital microscopy of the rat cremaster during acoustic exposure. Male rats of the Wistar strain weighing between 150 and 300 g were used.

2.

A circular aluminum platform, supported on 4 legs, fabricated in order to raise the tissue from the bottom of the tank and minimize standing waves. Standing waves, potentially caused by the ultrasound beam being reflected back to the same location in the tissue, could create an unknown increase or decrease in the exposure.

3.

Regions of the rat cremaster muscle imaged before and after exposure to ultrasound at 1000 Hz PRF (a, b, c, d) and 10 Hz PRF (e, f, g, h). A binary mask based on subtraction of post-exposure image from the pre-exposure image was produced (c and g). The count of white pixels on the binary mask with respect to the total number of pixels gives the percent area of extravasation. The mask was superimposed with post-exposure image (d and h) for better visualization of regions counted as extravasation regions. Pre-exposure images (a and e) were compared with post-exposure images (b and f) for the operator assessment of extravasation. Areas of extravasation are indicated by dashed boundary lines in (b) and (f). There is significantly greater damage at 10 Hz PRF than at 1000 Hz PRF ($p < 0.01$).

4. Acoustic emission presented as a function of pulse number (a) and total exposure duration (b). The RIPS associated with 10 Hz PRF is greater and more sustained than the corresponding RIPS at 100-, 500-, and 1000 Hz PRF, which reach noise levels in 20 pulses or fewer.

5.

Tissue damage due to the insonification of microbubbles within the vasculature of the rat cremaster, presented as area of extravasation. The area of extravasation, calculated as a percentage of the total area exposed, is shown for various PRF's. Values are expressed as mean and standard deviation. Damage is 16% ($\pm 12\%$ SD) at 1000 Hz PRF, 17% ($\pm 11\%$ SD) at 500 Hz, 14% ($\pm 9\%$ SD) at 100 Hz, and 60% ($\pm 15\%$ SD) at 10 Hz. Damage for 1000-, 500-, and 100 Hz PRF's is statistically similar, whereas, it is significantly higher for the 10 Hz PRF ($p < 0.01$). Operator assessed damage (closed markers) is compared with an automated approach (open markers).

6.

The correlation of tissue damage (area of extravasation) to acoustic emission (cumulative RIPS). CRIPS was used as the emission indicator since damage, measured at the end of each exposure, was a cumulative effect from all the pulses. There is good correlation between tissue damage and acoustic emission ($R^2 = 0.75$). Dashed lines in the figure split the extravasation-emission relationship into four quadrants.

7.

Scatter plot of tissue damage (area of extravasation) as a function of the peak rarefactional pressure (P_r). There is no visible damage at $P_r \leq 0.85$ MPa. Damage, however, is observed at $P_r \geq 1.0$ MPa and increases with increasing acoustic pressure. The least squares fit of the increasing trend has a correlation coefficient of 0.98.

Summary of the number of exposures performed at each peak rarefactional pressure for the pressure amplitude threshold study

Peak Rarefactional Pressure (MPa)	Number of exposures
0.5	3
0.75	3
0.85	2
1.0	3
1.5	3
2.0	1