Monitoring bubble growth in supersaturated blood and tissue *ex vivo* and the relevance to marine mammal bioeffects

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Abstract: There have been several recent reports that active sonar systems can lead to serious bioeffects in marine mammals, particularly beaked whales, resulting in strandings, and in some cases, to their deaths. We have devised a series of experiments to determine the potential role of low-frequency acoustic sources as a means to induce bubble nucleation and growth in supersaturated *ex vivo* bovine liver and kidney tissues, and blood. Bubble detection was achieved with a diagnostic ultrasound scanner. Under the conditions of this experiment, supersaturated tissues and blood led to extensive bubble production when exposed to short pulses of low frequency sound.

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1. Introduction

Both mid-range (~1–10 kHz) and low frequency active (LFA, <1 kHz) sonar systems have been implicated in mass stranding events of cetaceans, predominantly beaked whales.[1–3] In a report from a recent workshop on beaked whale strandings, the following comment is made: "Participants agreed on two major findings: 1) gas-bubble disease, induced through a behavioral response to acoustic exposure, may be the pathologic mechanism and merits further investigation...."[4] The mechanism for this bubble growth is unknown, but may involve direct nucleation from sound sources, or through behavioral changes leading to bubble nucleation, i.e., decompression sickness.

Behavioral changes due to unexpected sound stimuli have recently been reported from right whales.[5] Although these cetaceans have not been associated with mass stranding events related to navy sonar systems, it is likely that other cetaceans will also undergo significant changes in behavior when subjected to high-intensity acoustic pulses. Rapid surfacing from a deep dive may lead to decompression sickness. In addition, it is known that exercising after diving can lead to decompression sickness in humans.[6] Analogously, abnormal extended activity resulting from sonar may induce decompression sickness in cetaceans.

To address the role of direct bubble nucleation in tissue by a sound pulse, it is worthwhile to discuss the bioeffects induced by diagnostic ultrasound systems, used routinely worldwide to image the progress of healthy as well as pathological conditions in the human patient. It is no surprise, then, to recognize that ultrasound-induced bioeffects in human tissue have been studied extensively. To this date, no repeatable effects of diagnostic ultrasound exams have been reported in the general literature. This paucity of observable bioeffects was at first surprising because the acoustic pressure amplitudes used in imaging devices are in excess of the threshold for bubble nucleation and growth, i.e., cavitation—the most likely ultrasound-induced

damage mechanism—even for the short pulses and high frequencies that are typical of these systems.[7] The probable reason that these bioeffects are absent is that the required nucleation sites for bubbles to occur are either absent or inactive.[8] However, marine mammals that regularly make deep dives develop a condition that would not normally be experienced in human studies—that of local levels of gas supersaturation.

When a diver or marine mammal remains at depth for an extended period of time, the dissolved gas concentration within its body fluids can significantly increase from the sea-level value of 100% to a higher equilibrium value. For example, Houser *et al.*[9] have used dive profiles of dolphins and whales and some simple gas diffusion laws to compute the expected "intramuscular nitrogen tension;" levels approaching 300% saturation are reported.

If a sound field of moderate amplitude impinges on a diver or marine mammal at depth, then the pressure fluctuates about the at-depth ambient pressure and any preexisting bubble will be driven into radial oscillation. An oscillating bubble in a supersaturated fluid tends to grow due to rectified diffusion.[10] Crum and Mao[11] confirmed that bubble growth can occur with low-frequency sound fields, even at moderate pressures, provided the appropriate conditions are met. Furthermore, once these bubbles are nucleated, further growth can occur via static diffusion from the supersaturated solution.

The long-term goal of this research is to determine under what conditions, if any, that acoustic signals can lead *directly* to the nucleation of microscopic gas bubbles (and their subsequent growth to macroscopic sizes) in supersaturated *in vivo* tissues. However, the first step towards that goal is to determine the parameter space in which supersaturated tissue mimicking phantoms and *ex vivo* tissues do not prematurely and spontaneously outgas, but yet can be made to nucleate microbubbles under the action of pulsed sound. Towards this end, we have performed a set of illustrative experiments on tissue mimicking phantoms, *ex vivo* tissues, and blood, under various levels of supersaturation.

Some definitions of terms are perhaps in order: By "nucleation" we do not mean the creation of a gas bubble that did not previously exist; rather we mean that a preexisting, stabilized, nucleation site has been "activated" so that it can grow by either rectified or quasi-static diffusion.[12] For example, it is known that lipid monolayers can stabilize a gas bubble against dissolution. If this stabilizing monolayer is disrupted by the sound source—through bubble oscillation—and permits gas diffusion, then bubble nucleation is said to occur

2. Experiments

Preliminary studies involved inserting tissues or blood in a pressure chamber, placing the chamber under compression (400–700 kPa, 40–70 m nominal diving depth) for a specified length of time, decompressing the chamber, verifying that spontaneous outgassing does not occur, applying low-frequency sound pulses to the contents, and examining the contents for bubble growth with a diagnostic ultrasound scanner or direct video imaging.

2.1 Blood experiments

Blood experiment protocols were as follows: Approximately 2 L of fresh bovine blood was mixed with 20 mL heparin to prevent clotting, and stored in the refrigerator until needed. Immediately prior to filling the pressure chamber with blood, it was diluted with two parts PBS (phosphate-buffered saline). The blood/PBS solution was placed directly into the experimental pressure vessel shown in Fig. 1 (fluid holding part is labeled "b"). The vessel was pressurized to 700 kPa for 1 h (measured O_2 concentration level in water for these values is 250%). A magnetic stir bar was used to accelerate saturation throughout the blood. Upon depressurization, the lid was removed and an ultrasonic imaging probe (Terason, with 10L5 scanning head) (labeled "c") was immediately partially immersed in the fluid to image bubbles. Under these specific conditions, no spontaneous bubble formation occurred; that is, there was no significant outgassing in the bulk.

Two diametrically opposed 37 kHz flat-faced PZT transducers (APC International Inc.) mounted within the vessel (labeled "a") insonified the contents. The imaging transducer recorded the echogenicity during insonation. Mm. 1 shows that even at low pressure levels

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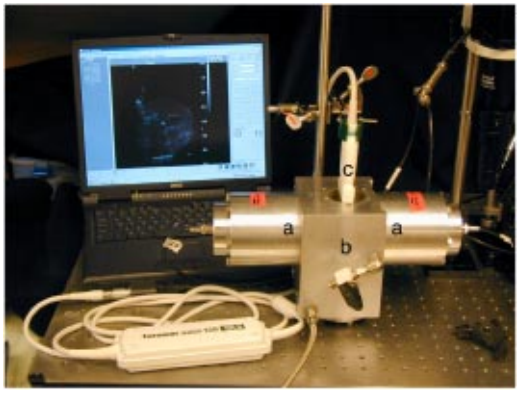

Fig. 1. View of pressure vessel after decompression and with the imaging probe in place. Diametrically opposed transducers (a) are used to generate (near-field) pressure levels up to 400 kPa in the vessel (b). Bubbles are viewed with diagnostic imaging (c), or a video camera (not shown).

(estimated to be ≈50 kPa, based on prior calibration), bubbles form between the transducer faces (approximately located at drawn lines). It is worth noting that the bubble nucleation threshold in *saturated* blood is much higher than 50 kPa; we were unable to nucleate bubbles with our system unless the blood was supersaturated.[13]

The Note that prior to sonication (initial segments of Mm. 1), no spontaneous bubble formation occurred. In addition, the ultrasound imaging probe itself (the top of the video is near the transducer probe, the bottom of the video is furthest from the probe) did not cause bubble nucleation. The sonication pressure levels used were low enough to prevent the blood from frothing.

Mm. 1. Video shows that supersaturated blood can be made to nucleate bubbles by ultrasound. File type "mov," 1 Mb.

2.2 Liver experiments

Liver experiment protocols were as follows: Bovine liver tissues were acquired from a local supermarket. They were sealed in a plastic container and stored refrigerated in PBS until needed. For the experiment, squares of length 3.8 cm were cut and placed into a beaker containing bovine blood with a magnetic stir bar. The beaker was placed into an ice bath (refrigeration was needed to prevent spoiling), which was then placed on top of a magnetic stir plate. The stirring was set to a stable rate, and this stack was sealed inside a (different) pressure vessel (not shown). The pressure was raised to 500 kPa for 3 h (the resulting gas saturation level within the liver tissue is unknown).

After depressurization, individual liver segments were removed and lowered into the pressure chamber of Fig. 1. A block of polyacrylamide gel was cut to fit inside the vessel and provide a pedestal on which the liver piece could sit within the active region between the transducers. The surrounding volume was filled with highly degassed water in order to prevent the solution itself from cavitating (nucleating bubbles) during sonication. In this way we were able to examine possible cavitation within the tissue itself. The ultrasound probe was then partially lowered into the solution for imaging.

Because the liver itself generates an image, it is difficult to observe the formation of individual bubbles, either in the presence or absence of sonication. To overcome this potential problem, we postprocessed the video and used the first image after the ultrasound pulse as a background image, subtracting it from every subsequent image in the movie. In this way, only changes in the images would show up. Mm. 2 shows an example of the postprocessed ultrasound video of a liver sample. Again, the top of the video is nearest the transducer probe, the bottom of

the video is furthest from the probe. The speckle is probably noise, due to enhancing the contrast.

Mm. 2. The interference pattern observed in the video is due to the tissue being insonated with 10 000 cycles at ≈400 kPa. Immediately afterwards, the video is dark, due to using this image as a background image. As the video progresses, hyperechoic echoes increase, both in size and number. It seems apparent that bubble growth is indeed occurring after the ultrasound pulse. No such bubble growth was observed in control experiments (where liver samples did not undergo ultrasound activation). Notice that towards the end of the video, a couple of bubbles are seen to dislodge and move upwards, towards the imaging probe. File type "mov," 1 Mb.

2.3 Kidney perfusion experiments

Kidney experiment protocols were as follows: *Ex vivo* porcine kidneys were obtained (under approved procedures at the University of Washington) with significant lengths to the renal artery and vein (≈1 cm), as well as the ureter still remaining.

The kidneys were placed in a blood-filled beaker with a magnetic stir bar (blood was obtained as described above). The beaker was then placed into an ice bath and, together with the magnetic stir system, lowered into a pressure vessel (same as liver study). The pressure was then raised to 400 kPa for 2 h.

Afterwards, the supersaturated (actual saturation levels are unknown) blood was withdrawn into a 60 mL syringe. A cannulus was placed onto the syringe, and the tip inserted into the renal artery of one kidney. The entire blood volume in the syringe was very slowly passed into the kidney, with the excess flowing out the renal vein. The perfused kidney was placed back into the beaker of blood and into the ice bath in the pressure vessel. The vessel was then repressurized for two additional hours.

The kidney was then removed from the blood in the beaker and cut in half axially. The top half of this kidney was placed into the pressure vessel between the transducers for sonication treatment. The bottom half was replaced into the beaker. As with the liver, a block of polyacrylamide gel was cut to fit inside the vessel and provide a pedestal on which the kidney would sit within the active region between the transducers. The imaging probe was then inserted in the top of the vessel as previously described. The kidney was sonicated with three acoustic pulses of 10 000 cycles, each 1 min apart.

Figure 2 illustrates the changes in echoes before (a) and after (b) sonication (and with an additional 30 min wait). The sonicated kidney appears to show evidence of bubbles in the ultrasound image. To examine this in more detail, the kidney was placed into an aluminum sample holder and set in alginate. The bottom half was also removed from the vessel and set in alginate. After the alginate was fully set, the control and sonicated kidney pieces were placed into a deep freezer at −65 °C. The pieces were left in the freezer for 3 days.

A microtome was used for slicing away the surface material of the frozen kidneys to expose tissues for optical imaging under a microscope. Sequential slices were removed and the exposed tissue was explored for signs of bubble formation. Corresponding slices were compared under the microscope. An example of the optical images we observed is shown in Fig. 3. There is a definite increase in the number and size of bubbles observed.

3. Conclusion

We examined the potential of bubble nucleation under sonication in supersaturated *ex vivo* bovine blood, liver, and kidneys. Parameter spaces were found such that bubble nucleation occurred only when the supersaturated tissues were sonicated. We found that bubbles can be nucleated from *ex vivo* tissues and blood that have undergone a compression-decompression sequence. Although sonication occurred at higher frequencies than found in active sonar systems, the physics of bubble nucleation from these acoustic pulses is not significantly different. These initial experiments should serve to stimulate further research on the potential for bubble nucleation within blood vessels and tissues in marine mammals. Recent evidence of the existence of osteonecrosis in sperm whales suggests that microbubble generation from deep-diving decompression may be not uncommon in marine mammals.[14]

We hypothesize that the sound source is a necessary but not sufficient condition for

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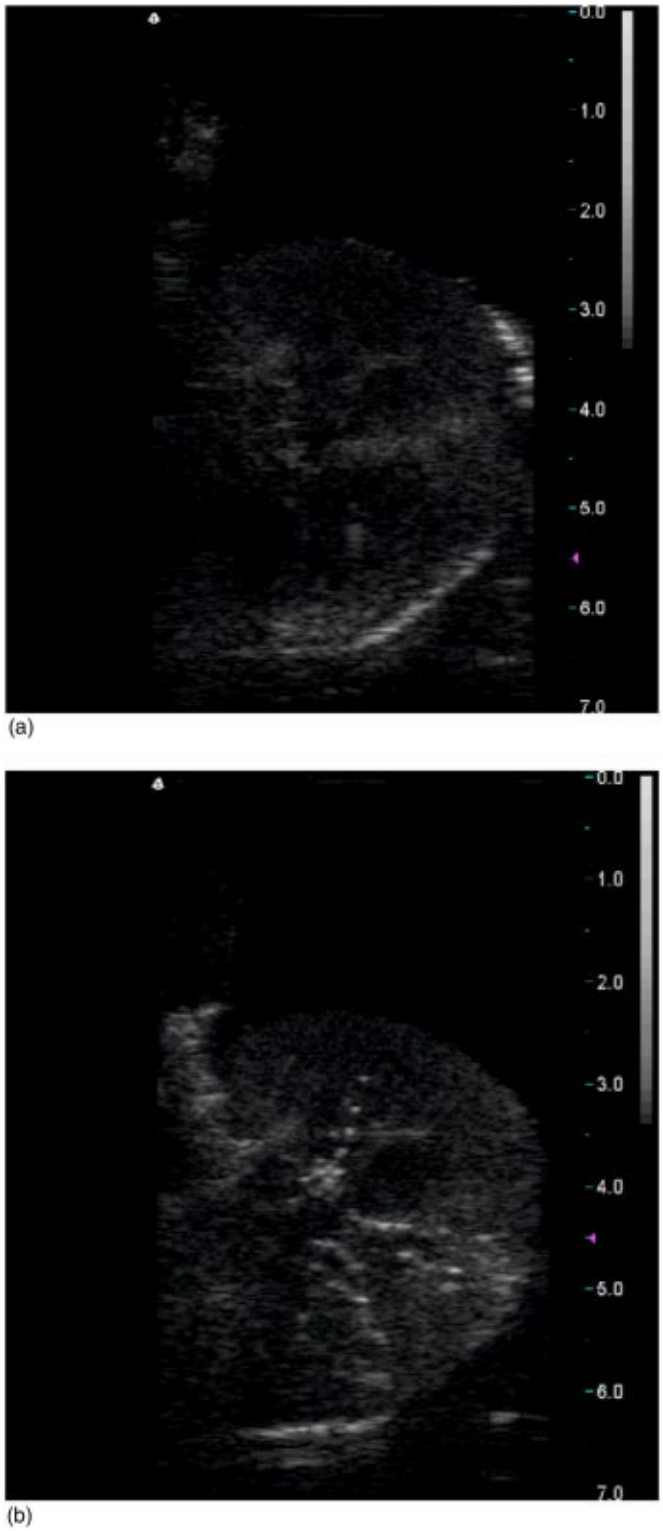

Fig. 2. (a) Ultrasound image of a kidney prior to ultrasound activation. (b) Ultrasound image of the kidney after sonication followed by a 30 min wait.

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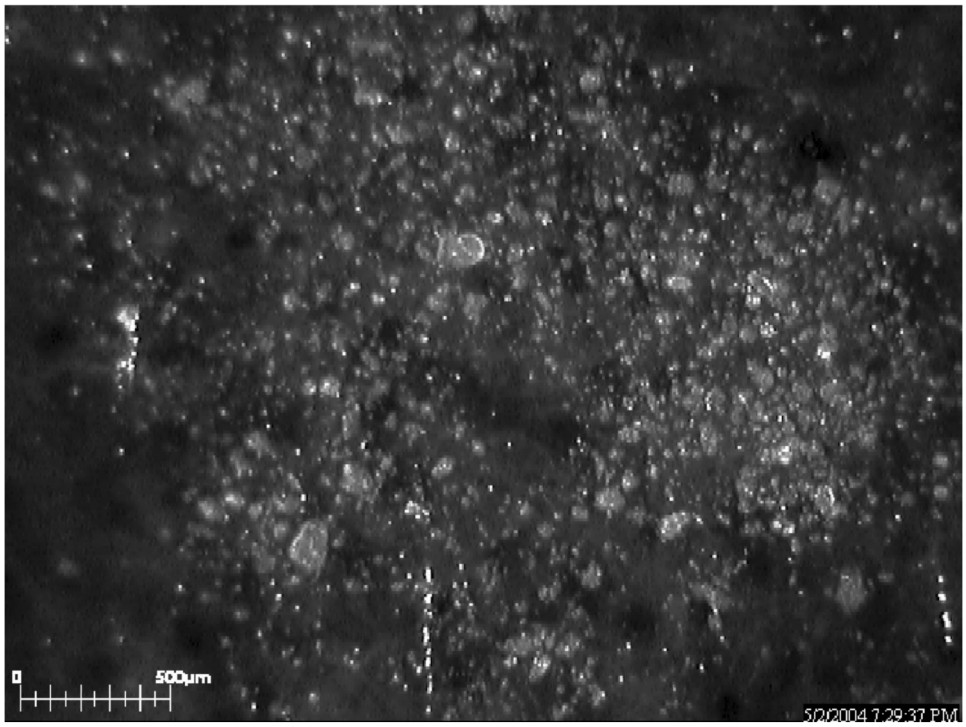
(a)



(b)

Fig. 3. Image of a slice of *ex vivo* kidney tissue under a microscope. (a) Kidney treated with supersaturation, but no ultrasound. (b) Kidney treated with supersaturation and ultrasound (see text for description). A significant number of bubbles have grown. In both cases, the tissue was left at atmospheric pressure for about 30 min after decompression before being frozen for slicing.

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bubble production. The fluid-filled spaces in the marine mammal must be supersaturated for macroscopic bubble growth to occur. We do NOT argue that these bubbles are produced by rectified diffusion; rather, we argue that the sound source causes previously stabilized, preexisting, microscopic gas bubbles to be activated; i.e., the stabilization mechanism is somehow disrupted, and the local supersaturation of body fluids leads to macroscopic bubble growth of these destabilized nuclei by quasi-static diffusion.

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