Microbubble detection following hyperbaric chamber dives using dual-frequency ultrasound

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Swan JG, Bollinger BD, Donoghue TG, Wilbur JC, Phillips SD, Alvarenga DL, Knaus DA, Magari PJ, Buckey JC. Microbubble detection following hyperbaric chamber dives using dual-frequency ultrasound. J Appl Physiol 111: 1323–1328, 2011. First published August 18, 2011; doi:10.1152/japplphysiol.01203.2010.—Venous gas emboli (VGE) can be readily detected in the bloodstream using existing ultrasound methods. No method currently exists to detect decompression-induced microbubbles in tissue. We hypothesized that dual-frequency ultrasound (DFU) could detect these microbubbles. With DFU, microbubbles are driven with two frequencies: a lower “pump” (set to the resonant frequency of the desired bubble size) and a higher “image” frequency. A bubble of the resonant size emits the sum and difference of the two transmitted frequencies. For this study we used a pump frequency of 2.25 MHz and an image frequency of 5.0 MHz, which detects bubbles of roughly 1–10 μm in diameter in a water tank. Four anesthetized swine were pressurized at 4.5 ATA for 2 h and decompressed over 5 min, inducing moderate to very severe VGE scores. Four sites on the thigh of each swine were monitored with DFU before and after the dives. A single mock dive was also performed. The number of sites returning signals consistent with microbubbles increased dramatically after the chamber dive (P < 0.01), but did not change with the mock dive. The increase in DFU signal after the chamber dive was sustained and present at multiple sites in multiple swine. This research shows for the first time that decompression-induced tissue microbubbles can be detected using DFU and that DFU could be used to monitor decompression-induced microbubbles at multiple sites on the body. Additionally, DFU could be used to track the time course of microbubble formation and growth during decompression stress.

decompression sickness; venous gas emboli

WHEN THE PRESSURE around the body is actively decreasing (decompression) or after decompression when nitrogen levels within the body are equilibrating with the surrounding pressure (decompression stress), air-filled bubbles can form in tissue and blood vessels as stored gases equilibrate with the surrounding environment. Venous gas emboli (VGE) are commonly observed during decompression stress, even for regulated dives (6). The VGE grade measured during decompression stress is often used as a marker of severity and to assess the adequacy of decompression sickness countermeasures (8). Both two-dimensional (2D) and Doppler ultrasound can readily detect VGE in the heart and/or major blood vessels (1, 15). If, however, VGE bubbles can be detected in the heart and/or major blood vessels, it is likely that bubbles are forming in other locations throughout the body as well. Microbubbles (in the ~1–10 μm diameter range) are hypothesized to form in tissues during decompression stress before the larger VGE bubbles (9). Microbubble detection in tissue locations outside of the heart/blood vessels could potentially offer more information about the severity of decompression stress, while detection of smaller microbubbles may allow for early markers of decompression stress.

Currently, tissue microbubble detection is limited by several factors. Tissue microbubbles are likely very small (in the ~1–10 μm diameter range) and are thought to be widely distributed initially during decompression (9). This would make them hard to distinguish from other ultrasound reflectors in tissue. Although standard ultrasound can detect microbubble ultrasonic contrast, this detection depends on the microbubbles as a group increasing ultrasound reflections and, therefore, increasing ultrasonic contrast. Standard ultrasound techniques cannot reliably distinguish small numbers of microbubbles in a background of multiple other ultrasound reflectors. Dual-frequency ultrasound (DFU), which generates ultrasound returns from bubbles but not other linear ultrasound reflectors, may offer a new way to detect microbubbles in tissue (16).

The dual-frequency technique used in this work has been described elsewhere (3, 5). Briefly, DFU is a low-mechanical index (MI) nonlinear ultrasound technique that transmits two frequencies instead of one (13). A lower “pump” frequency, f0, is used to drive bubbles at their resonant frequency. As the resonant frequency of a bubble is inversely proportional to the bubble diameter, the pump frequency can be adjusted to target bubbles of various sizes (12). While factors such as the shape of the microbubble and the microenvironment (attachment to tissue surfaces, interstitial pressures) can affect the resonant frequency of the bubble, overall a relationship between resonant frequency and bubble diameter is still expected to hold true. A second, higher “image” frequency, fi, is transmitted as well. The resulting nonlinear oscillations, under the influence of both f0 and fi, cause the bubble to emit the sum, f+ = f0 + f0, and the difference, f(−) = f0 − f0, of the two driving frequencies. Detection of sum and difference signals indicates the presence of bubbles at the resonant size. In previous work we demonstrated that DFU can be used to size microbubbles in vitro (2) and that in vivo it can detect very small microbubbles in tissue produced by exercise (16). No other technique exists to confirm the presence of tissue microbubbles detected by DFU, but the presence of microbubbles is the best explanation for the signals we have collected in these previous studies.

The purpose of this study was to demonstrate whether DFU could detect tissue microbubbles during decompression stress. If these microbubbles can be detected, this offers 1) the ability to monitor individuals exposed to decompression at many sites in the body and 2) a new research tool for assessing the...
location and strength of microbubble signals at tissue sites throughout the body.

METHODS

Animals. All experiments were performed on 12-wk-old, 20-kg female swine (Parson’s Farm, Westhampton, MA). The Dartmouth Institutional Animal Care and Use Committee approved the research protocol.

Anesthesia protocol. Swine were initially anesthetized using 20 mg/kg ketamine and 0.05 mg/kg xylazine. Atropine (0.04 mg/kg) was given to reduce oral and nasal secretions. Swine were then intubated and ventilated (1 l/min 100% O₂ + 2% isoflurane) using a volume-cycled ventilator while baseline measurements were taken. The baseline period, which included oxygen prebreathe, was set at 45 min to standardize denitrogenation times between subjects. A pulse oximeter was placed on the ear for monitoring O₂ saturation and pulse. O₂ saturation was kept above 90% for the entire time the animal was on anesthesia.

Following the baseline period, swine were taken off inhalation anesthesia, but the endotracheal tube was left in place to maintain airway patency. Anesthesia was maintained in the hyperbaric chamber by bolus injections of pentobarbital sodium (total dose = 20 mg/kg) administered through two intravenous lines placed in ear veins. At the conclusion of the hyperbaric exposure, swine were removed from the chamber, reconnected to ventilator, and returned to isoflurane anesthesia (∼1 3/min 100% O₂ + 2% isoflurane) while post-dive data were collected.

Dive chamber protocol. Swine were at 1 ATA air while baseline measures were taken. Baseline measurements were taken every 2–3 min starting no later than 30 min prior to hyperbaric exposure. Following the baseline period, the swine were placed in a polycarbonate enclosure, which was then slid into the hyperbaric chamber. The swine were compressed to 4.5 ATA at a rate of 0.6 atm/min. Swine were held at 4.5 ATA for a total of 120 min. After this time period, the swine were decompressed to 1 ATA pressure at a rate of 0.6 atm/min. The swine were then removed from the chamber. Post-dive measurements were obtained following removal of the swine from the chamber. As soon as possible following the return of the swine to 1 ATA (usually 1–2 min), post-dive measurements were collected. For the sham dive, the swine experienced the exact same sequence and timeline. Several scan cycles were completed during the baseline period. Similarly, multiple scan cycles, cycling through the four thigh sites and the right ventricle. A mini-ultrasound machine (Philips iU22 scanner) was used to ensure that no potential strong ultrasound reflectors (i.e., bones or joints) that could generate false-positive signals were in the measurement volume. Major blood vessels were also avoided. The DFU transducers were arranged so that the beams intersected at a depth of 2.5 cm, with a calculated measurement volume of ∼0.4 ml. VGE grade was assessed using 2D ultrasound to image the right ventricle. Images were stored and analyzed after the trial was completed. Each scan cycle consisted of cycling through the four thigh sites and the right ventricle. A minimum of two scan cycles, scanning each site in the same order, were completed during the baseline period. Similarly, multiple scan cycles, scanning each site in the same order as baseline, were completed following chamber dives. Post-dive measures typically began 1–2 min after exiting the hyperbaric chamber. Data scan cycles continued until skin mottling consistent with skin bends (cutis marmorata) appeared or 1 h had elapsed. Cutis marmorata was used as an end point because previous experience has shown that once this point is reached, gas in the skin hinders ultrasound transmission, making it difficult to obtain measurements.

VGE grading. Images from the right ventricle obtained following the chamber dives were stored and analyzed at a later time point. Swine were given a score between zero and five using the criteria outlined in Brubakk and Eftedal (4). A single observer, who learned the technique by reading the literature and receiving training from an experienced observer, did all grading from the ultrasound recordings. Although the grader was relatively inexperienced, experience has been shown to play a small role in accurate VGE determination using this technique (7). For one swine, the right ventricular images were not saved, and the estimated VGE scores performed visually and recorded during the experiment were used.

DFU signal collection. The dual-frequency technique used in this work has been described elsewhere (3, 5). To determine the difference signal, we performed a fast-Fourier transform (FFT), which is a mathematical algorithm expressing data in the frequency domain of the time-dependent electrical signal returned via the receiver transducer. We recorded the amplitude of the difference signal, f_v (as well as the average background noise of the FFT). The amplitude of the difference signal was reported in the results in units of decibels relative to the background noise (db re: noise floor) amplitude.

Statistical analysis. Five swine were used for this experiment: four were exposed to decompression stress and one was not. An individual site was considered positive for the presence of microbubbles if the mean of the DFU measurement (amplitude of the difference frequency component) taken post-chamber dive was statistically greater than the mean baseline DFU data as determined by a two-tailed t-test. Each site was compared with itself. After the determination of whether a site was positive for microbubbles, positive sites were tallied for all swine and grouped into their respective time period: baseline, post-dive, or mock dive. Comparison between the three time periods was then made using a χ² analysis to determine which period produced a statistical increase in microbubbles.

To determine a main effect for decompression and time, a repeated-measures ANOVA was used to compare the multiple measurements made at each site pre-dive and post-dive. All DFU data were grouped in 2.5-min intervals. For example, data collected in the first 2.5 min after exiting the chamber were averaged and labeled as time 0. The final two DFU measurements immediately preceding entry into the hyperbaric chamber were used as the baseline. All significant interactions were analyzed using the Fisher’s post hoc analysis. Data on the amplitude of the received pump and image frequencies were also collected. To compare pump and image frequency amplitude over time, a two-way ANOVA was used.

Statistical significance is achieved when P < 0.05. All data are presented as average ± standard deviation.

RESULTS

All swine survived their respective hyperbaric or mock dive. Each swine that experienced hyperbaric pressurization had significant VGE in the right ventricle immediately after return to normobaria. VGE scores (Fig. 1) demonstrate that each dive produced moderate (VGE = 3; swine 3) to high VGE (VGE score = 4–5; swine 1, 2, and 4). Data collection was terminated before 1 h had elapsed in two of the swine because of progressive cutis marmorata (swine 2 and 4). These two swine also demonstrated high VGE (scores = 4–5). The mock-dive swine did not have any VGE.

DFU data from the last two baseline measurements prior to the chamber dives were compared with the first two measurements following hyperbaric exposure. These time periods were used because this formed a complete dataset that included all 16 sites. These data show that decompression stress caused a
a statistically significant increase in microbubble detection compared with baseline ($P < 0.01$; Fig. 2). During baseline, three measurements showed the presence of microbubbles, while 37 did not (7.5%). Following decompression, 11 sites showed microbubbles, while 21 did not (34%). The mock dive showed one positive measurement and seven negative measurements (12.5%).

Figure 3 shows the compiled data (dB re: noise floor) taken from all swine at all sites. The data show a significant increase in the difference signal following the chamber dives, suggesting an increased occurrence of microbubbles. The DFU signal post-dive was statistically greater compared with both baseline and the mock dive ($P < 0.01$).

The time course for the microbubble signal (dB re: noise floor) during decompression stress for each individual swine is shown in Fig. 4. Microbubbles typically started to increase ~3–4 min after surfacing and typically peaked by minutes 12–13. Examination of the individual swine graphs (Fig. 4) shows that the swine that did not last the entire post-dive data collection period (swine 2 and 4) had an initial increase in DFU signal, which then returned to baseline. The two swine that were able to last the entire post-dive collection period (swine 1 and 3), showed a steady DFU signal, which was above baseline the entire post-dive collection period. One site (swine 3, site 2) showed a high baseline signal.

Large concentrations of microbubbles can strongly attenuate the amplitude of the driving (pump and image) ultrasound signals. This can decrease the measured DFU return (owing to the attenuation of the ultrasound), which may explain the decrease in DFU signal with time measured for swine 2 and 4. To ascertain whether ultrasound transmission was affected by the study, the amplitudes of the pump and image components of the received signal for each data collection point were analyzed. The data from the swine experiments that ended prematurely (due to visible cutis marmorata) showed attenuation of the pump and image frequencies. This decrease was not seen in the two swine that completed the entire protocol (Fig. 5). This decrease was not statistically significant ($P = 0.11$), most likely due to the small sample size ($n = 2$ for both groups). However, these data, combined with the appearance of cutis marmorata, suggest that increased gas in the tissue attenuated ultrasound transmission.

**DISCUSSION AND CONCLUSIONS**

These data show that signals consistent with microbubbles (in the ~1–10 μm diameter range) can be detected in tissue following hyperbaric chamber dives. As mentioned previously, it is not possible to confirm independently, as no other tech-
Fig. 4. Individual swine graphs for each specific data collection site as a function of time. Swine 2 and 4 did not complete the entire post-dive data collection period, since they developed cutis marmorata.

Fig. 5. Graphs representing pump and image return levels as a function of time. Swine 3, site 5 is a representative sample of pump and image return levels from a swine that completed the entire post-dive data collection period, while the graph on the right is representative of data from a swine that did not complete the entire period. Swine 2, site D5 shows no decrease in either the pump or the image return signal, while the graph on the right shows a marked decrease in both the pump and the image return frequencies at the end of the testing period. These data, combined with the appearance of cutis marmorata, suggest that increased gas in the tissue attenuated ultrasound transmission.

Although microbubbles have been hypothesized to form with decompression stress (9), the technology had not existed previously to measure them outside of primary vasculature. Using DFU, we were able to detect microbubbles in leg tissue, which includes muscle, adipose, nerve, and vasculature. Being able to track the time course of microbubble appearance and disappearance outside of major blood vessels, areas difficult to image with traditional ultrasound, following hyperbaric chamber dives could be a major benefit for the study of decompression-related injuries.

In this study, microbubbles appeared promptly following decompression to normobaric conditions. In the two animals where measurements were available for the entire post-dive data collection period, microbubbles were observed the entire time. In the two swine that did not last the entire post-dive data collection period, microbubbles were observed immediately following removal from the chamber, but tapered off to baseline levels ~15 min before the study was terminated. There are three possibilities for this occurrence. Microbubbles may have diminished in these animals, the bubbles may have grown so that they were no longer within the resonance range of the pump frequency being used, or the increase in microbubbles was so great that the forcing ultrasound was attenuated, leading to a decreased DFU signal. Examining the return signal of both the pump and image frequencies showed that there was a trend for the return signal to be diminished, suggesting a large increase in microbubbles attenuating the forcing ultrasound (see Fig. 5). These data, combined with the clinical observation of cutis marmorata, strongly suggest that the reason for this finding was high levels of gas within the tissue, which attenuated the ultrasound signals.
One site was positive for bubbles (swine 3, site 2) at baseline. Several possibilities exist for this. One is that bubbles were present in this area. There is considerable evidence that bubbles exist in tissue normally and that their numbers can be increased with movement. It is possible that this particular site had a high baseline level of bubbles. Another possibility is some other source of tissue nonlinearity is causing the ultrasonic signals to mix. For example, a strong ultrasound reflector, like a bony interface, could lead to nonlinear mixing of the signals. The sites were chosen so that strong reflecting surfaces were avoided, but it is possible that at this site, there may have been a strong reflector partially within the ultrasonic beam. Since the measurements were made at the same sites both before and after the dive, the presence of a strong reflector at one site would not influence the overall findings from the study (i.e., each site serves as its own control).

**Significance.** These results show that DFU could offer a new way to monitor individuals experiencing decompression stress. Currently, either Doppler or 2D ultrasound measurements of the heart and great vessels are used to detect VGE. But, cardiac imaging is often limited by finding acceptable ultrasonic “windows” between ribs in the chest. DFU offers the potential to detect microbubbles with specificity in a variety of body sites aside from the heart and great vessels. In this study, the thigh of the swine was used, but it is likely that any other tissue site accessible to ultrasound could be used as a potential bubble detection site. This could offer considerable flexibility in monitoring for bubbles after dives or during altitude exposure. Since the presence or absence of cardiac bubbles is sometimes used as an index of decompression stress, it is possible that DFU may offer a more robust way to make bubble measurements since detection sites aside from the heart could be used.

This technology could also be used in decompression sickness research. By providing information about bubble size, it may be able to track the change in the size distribution of bubble populations over time. These data would be useful in validating microbubble growth models. Also, DFU can detect much smaller bubbles than can be detected with specificity using Doppler or imaging ultrasound. Other techniques currently being developed for microbubble detection include ultrasound-based techniques such as Second Order Ultrasound Field (SURF) imaging (11) and optical techniques, such as Optical Coherence Tomography (10). SURF imaging is a dual-frequency, ultrasound-based technique similar to DFU that shows significant promise for microbubble detection but which has not yet been demonstrated to detect endogenous microbubbles in humans. Optical techniques have penetration of only 1–2 mm in tissue, which limits their application in humans. Using DFU technology, bubble formation may be detectable earlier or more reliably than with existing techniques. In the current study, all animals had VGE shortly after the chamber dives, so microbubbles in the thigh were detected at the same time that VGE were also present. Whether the two are related remains to be seen. Due to the logistics of the dive protocol, detecting tissue microbubbles prior to VGE was not possible. The possibility exists, however, that DFU may be able to detect microbubble formation prior to the appearance of VGE or at times when VGE are not present.

**Limitations.** The difference signal produced using the DFU technique is a consequence of nonlinear interactions within the tissue. At the power levels used in this study, microbubbles are the most likely source of those nonlinear interactions. Particularly in the post-hyperbaric exposure period we believe it is reasonable to assume the signals are due to microbubbles, since the decompression clearly produced bubbles in the bloodstream, and the signals did not increase in the sham dive. We cannot absolutely exclude the presence of other, previously unrecognized, sources of nonlinear interactions that could also produce difference signals.

With our current DFU device, the exact anatomic location of the bubble signal cannot be ascertained. It is not possible to state if the microbubbles detected were within muscle, fat, blood vessels, or interstitial fluid.

Another limitation of the current study is that all decompressed swine had VGE scores greater than three. So it is not possible to state whether DFU could detect microbubbles prior to the appearance of VGE or in circumstances where VGE are not detected. Further research is needed to determine the time course of microbubble formation and to ascertain whether DFU can detect microbubbles in settings where VGE are not found.

This study also used a single-pump frequency. This meant that only a narrow size range of microbubbles (estimated to be between 1 and 10 μm diameter based on the free bubble resonant frequency) were detected. Future studies could use a range of pump frequencies to detect a larger size range of microbubbles that may appear in tissue during decompression stress. Additionally, the time course of the microbubble size distribution could be tracked over time.

**Conclusion.** Our results indicate that microbubbles form at multiple tissue sites following decompression and persist for at least 1 h. Microbubbles were not detected above baseline values during the sham dive. The ability to measure microbubbles in tissue may provide a new way to monitor individuals during decompression stress and to answer questions about microbubble growth, size, and location.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**