Microbubbles are detected prior to larger bubbles following decompression

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Submitted 16 October 2013; accepted in final form 14 January 2014

Microbubbles are detected prior to larger bubbles following decompression. J Appl Physiol 116: 790–796, 2014. First published January 16, 2014; doi:10.1152/japplphysiol.01156.2013.—Using dual-frequency ultrasound (DFU), microbubbles (<10 μm diameter) have been detected in tissue following decompression. It is not known if these microbubbles are the precursors for B-mode ultrasound-detectable venous gas emboli (bmdVGE). The purpose of this study was to determine if microbubbles could be detected intravascularly postdecompression and to investigate the temporal relationship between microbubbles and larger bmdVGE. Anesthetized swine (n = 15) were exposed to 4.0–4.5 ATA for 2 h, followed by decompression to 0.98 ATA. Microbubble presence and VGE grade were measured using DFU and B-mode ultrasound, respectively, before and for 1 h postdecompression, approximately every 4–5 min. Microbubbles appeared in the bloodstream postdecompression, both in the presence and absence of bmdVGE. In swine without bmdVGE, microbubbles remained elevated for the entire 60-min postdecompression period. In swine with bmdVGE, microbubble signals were detected initially but then returned to baseline. Microbubbles were not detected with the sham dive. Mean bmdVGE grade increased over the length of the postdecompression data collection period. Comparison of the two response curves revealed significant differences at 5 and 10 min postdecompression, indicating that microbubbles preceded bmdVGE. These findings indicate that decompression-induced microbubbles can 1) be detected intravascularly at multiple sites, 2) appear in the presence and absence of bmdVGE, and 3) occur before bmdVGE. This supports the hypothesis that microbubbles precede larger VGE bubbles. Microbubble presence may be an early marker of decompression stress. Since DFU is a low-power ultrasonic method, it may be useful for operational diving applications.

ultrasound; decompression sickness; dual-frequency ultrasound; venous gas emboli

FOLLOWING DECOMPRESSION, VENOUS gas emboli (VGE) may appear in the bloodstream (10). These emboli are readily detected by B-mode ultrasound in the chambers of the right heart. With moderate decompression stress, VGE often appear as occasional bubbles initially (VGE Grade 1) and then can progress over time to large amounts of bubbles (1 bubble every cm², VGE Grade 4) or even complete whiteout (VGE Grade 5) as described by Brubakk and Efetal (6). VGE grade correlates positively with the rate of ascent. Although VGE grade does not reliably predict decompression sickness (DCS) symptoms, it is used as a marker of decompression stress. Efetal et al. proposed that bubble detection using B-mode ultrasound imaging could be used as a tool to assess the safety of decompression procedures (11). If, for example, a particular prebreathing strategy was effective at preventing the appearance of VGE, then this procedure could also be effective at preventing decompression sickness.

Larger VGE bubbles likely begin as smaller microbubbles, and so smaller microbubbles should appear in the vascular system prior to larger VGE bubbles (3, 17, 22, 24). In theory, if microbubbles could be detected at the microbubble stage, this might provide earlier detection of decompression stress than standard VGE detection provides. Such a marker could potentially be used to prevent decompression sickness, by giving the diver advance warning of bubble formation and allowing for countermeasures to be employed (e.g., by using longer or deeper decompression stops).

Until recently, the technology to detect microbubbles at physiological concentrations has not been available. In previous work, we have shown that dual-frequency ultrasound (DFU), which generates ultrasound returns from bubbles but not other linear reflectors, can detect microbubbles. DFU can be used to size microbubbles in vitro (4), detect small microbubbles in tissue produced by exercise (23), and detect bubbles in tissue after decompression (21).

The goal of the present study was to determine if DFU could detect decompression-induced microbubbles in the vascular system and whether these bubbles would precede the appearance of B-mode ultrasound-detectable VGE (bmdVGE). We established the sensitivity of DFU for detecting ultrasonic contrast agent (Definity, Bristol-Myers Squibb, N. Billerica, MA) in an in vitro study. Also using ultrasonic contrast, we confirmed that the DFU could detect injected microbubbles at vascular sites. We then monitored decompression-induced bubbles at three vascular and one extravascular tissue sites in 15 swine exposed to decompression and one sham dive. We hypothesized that DFU could detect microbubbles at both vascular and tissue sites and that this detection would precede bmdVGE.

METHODS

The Dartmouth Institutional Animal Care and Use Committee approved the animal-related research protocols.

DFU signal collection. The dual-frequency technique used to detect microbubbles has been described elsewhere, so it will be described only briefly here (5, 7). Two continuous wave frequencies of ultrasound (2.25 and 5 MHz) interrogate the area to be measured, and the return signal is simultaneously recorded. The presence of microbubbles is determined by the strength of the returning signal at the difference of the two interrogating frequencies (e.g., 2.75 MHz). An increase in the difference signal from baseline was used as a measure of microbubble presence. To determine the difference signal, we performed a fast-Fourier transform (FFT), a mathematical algorithm expressing data in the frequency domain, of the time-dependent electrical signal returned via the receive transducer. We recorded the amplitude of the frequency spectrum at the difference frequency (the “difference signal”) as well as the average noise of the spectrum near the difference frequency (calculated as the median signal level within a few 10,000 Hz on either side of the difference frequency). The

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amplitude of the difference signal is reported in the results section both in units of decibels relative to the background noise amplitude (signal-to-noise ratio, dB SNR) and as decibels relative to the mean signal amplitude collected during the baseline measurements (signal-to-baseline ratio, referred to here as dB SBR). The dB SNR metric is an absolute measurement as the noise floor of the system remained effectively constant for all experiments, while the SBR metric is a relative measure designed to capture changes between the baseline and positive measurements. Generally, the SBR is 1–3 dB lower than the SNR.

**DFU sensitivity study.** To assess the sensitivity limit of DFU, we compared the returned difference signal from decreasing concentrations of microbubbles to the return from a control solution with no microbubbles. Ultrasound contrast agent (Definity; bolus injection = 10 μL/kg as per manufacturer’s instructions) was injected according to the manufacturer’s specifications and diluted to specified concentrations in degassed saline. The control solution was saline that contained no ultrasound contrast agent and had been degassed to eliminate any bubbles. Approximately 70 mL of the test concentrations were placed in a plastic test vial. A holder was built to hold both the test vial and the DFU transducers in place to minimize any variation due to positioning. The holder, along with the transducers and test vial, were then placed into an aquarium where the tests were conducted. The aquarium was filled with mineral oil and lined with acoustically suppressing paint (Aptflex F28, Precision Acoustics, Dorchester, UK) to minimize standing waves. The returned difference signal was measured in triplicate for each concentration and in rotational order. Comparisons were made between the control solutions and each microbubble concentration.

For comparison, we determined the sensitivity for the B-mode ultrasound device (Ultrasonix, Sonix RP) we used in our swine studies using the same procedure. The ultrasound transducer was placed into the aquarium with the test vials. Each vial was measured in triplicate at 6.6 MHz and 10 MHz settings; all ultrasound settings were kept identical for each measure within each transducer setting. Grayscale pictures were taken and analyzed for pixel intensity inside the test vial (Image J). Comparisons were made between the control solutions and each microbubble concentration.

**Detection of microbubbles at vascular sites.** To verify that the DFU unit could detect microbubbles in the vasculature, ultrasound contrast agent (Definity; bolus injection = 10 μL/kg as per manufacturer’s instructions) was injected into ear vein IV line and the vascular return difference signal was measured. All experiments were performed using Doppler ultrasound and marked. DFU data were collected at the femoral, brachial, and carotid vascular bundles. These sites were located prior to injection (time 0 min). Video from the right ventricle of 0.6 ATA/min (Fig. 1). After this time period, the swine were decompressed to 0.98 ATA pressure at a rate of −0.6 ATA/min. The swine were then removed from the chamber, the pass-through catheter was disconnected, and the chamber pulse oximeter was removed. As soon as possible following the return of the swine to 0.98 ATA, positive measurements were begun (time = 0 min). For the sham dive, the swine experienced the exact same sequence and time course of events as outlined above. The sham dive, however, did not involve compression/decompression. The sham swine was put on anesthesia and placed in the hyperbaric chamber but remained at 0.98 ATA pressure for the 120 min of dive time.

**Pre and post data collection protocol.** Data were collected before the dive and following decompression. Difference signals were collected at four anatomic sites: three vascular sites (carotid, brachial, and femoral vascular bundles) and one tissue site (lateral biceps femoris). The tissue site was used since previous research had shown that microbubbles could also be detected in tissue. Each site was located and scanned using a clinical B-mode ultrasound machine (Ultrasonix, Sonix RP) to ensure that no potential strong ultrasound reflectors (i.e., bones or joints), which could generate false positive signals, were in the measurement volume. The vascular sites were located using B-mode and verified using Doppler ultrasound. Baseline difference signals were obtained over ~5 min (300 measures). Post-decompression, difference signals were collected at each site for ~30 s (30 measures), cycling through each site multiple times in a fixed order. Each site was measured approximately once every 4–5 min. Multiple scan cycles were completed for 1 h after decompression or until skin mottling consistent with skin bends (cutis marmorata) appeared over the measurement sites. Cutis marmorata at the measurement sites 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.** VGE grade was assessed using B-mode ultrasound (Ultrasonix, Sonix RP) to image the right ventricle. VGE grade was assessed prior to entering the hyperbaric chamber and every 5 min postdecompression, beginning as soon as possible after the swine exited the chamber (time = 0 min). Video from the right ventricle obtained following the chamber dives were stored and analyzed at a later time point. Swine were given a score between 0 and 5 using the criteria outlined in Brubakk and Eftedal (6).

**Statistical analysis: Sensitivity study.** To determine the sensitivity threshold of DFU, the difference signal measurements from all three trials were averaged and compared with the saline-filled target control using a two-way t-test. Similarly, the B-mode ultrasound sensitivity threshold was determined by averaging the pixel intensity from all three trials and comparing each trial to the saline-filled target control using a two-way t-test.

**Statistical analysis: Dive study.** Swine were divided into two groups based on the level of VGE present postdecompression. Swine with VGE ≥ 3 (at least one bubble every heart cycle) at any time point...
were placed into the VGE High group. Swine with VGE < 3 at all time points were grouped in the VGE Low group.

Prior to statistical analysis, difference signal data (in dB SBR), both pre and postive, were first organized into 5-min epochs for each swine. The average for each epoch was then computed across swine. To determine if microbubbles were increased over baseline, normality of data was first assessed using the Kolmogorov-Smirnov test, with \( P < 0.05 \) indicating a nonnormal distribution. This test showed that our difference signal data were not normally distributed so the nonparametric Friedman test was used to determine differences between baseline and any postdecompression time point within each VGE group. If the Friedman test indicated a significant difference in the data set, a post hoc comparison using the Wilcoxon signed rank test was run to determine which time epochs were significantly different. Time epochs were considered statistically different if their \( p \) value was less than \( p \) value adjusted for multiple comparisons using the Bonferroni approach (\( n = 13 \) comparisons; critical \( p \) value = 0.003).

All data are presented as average ± standard deviation, except for VGE data which are presented as medians. \( P \) values are stated when appropriate.

RESULTS

**DFU sensitivity study.** The sensitivity of DFU was 10 microbubbles per mL (\( P < 0.01 \); Fig. 2, top). This concentration may be an absolute physical limit on the sensitivity of any DFU-based nonlinear technique. Modeling of the effective nonlinear acoustic coefficient for difference signal generation by microbubble-containing liquids [following the technique used in (13)] shows that the added nonlinearity due to the microbubbles (relative to the host liquid) quickly becomes very small below concentrations of roughly 100 microbubbles/mL. Attempts to raise the SNR to improve sensitivity further (e.g., higher ultrasound intensity, increased averaging) always uniformly increased the measured difference signal independent of the target (e.g., saline control or microbubble solution). The sensitivity for the 6.6-MHz B-Mode imaging ultrasound in the same experimental facility was \( 10^4 \) microbubbles/mL, while the sensitivity for the 10-MHz B-mode imaging ultrasound was \( 10^2 \) microbubbles/mL (Fig. 2, bottom).

**Detection of injected microbubbles in the vasculature.** Injection of ultrasound contrast agent was detected in all vascular sites (Fig. 3). The time to peak signal was \( \sim 45 \) s using DFU, compared with the manufacturer’s published time to maximum intensity using B-mode ultrasound of 1.13 min (1). Following the measured peak, the signal decayed to baseline over a period of \( \sim 7 \) min. This time was similar to the published decay times for Definity (1). These data verify that DFU is capable of detecting circulating microbubbles at vascular sites.

**Postdecompression.** A total of 15 swine were exposed to the dive profile shown in Fig. 1, plus one swine undergoing a mock dive. All swine were able to complete the full hour of data acquisition following decompression. Cutis marmorata (aka skin bends) was noted in three of the swine following decompression but was not located at the measurement sites and therefore did not interfere with data collection. Eight swine underwent compression to 4.0 ATA, of which two developed VGE >3. Seven swine underwent compression to 4.5 ATA, of which three developed VGE >3. Figure 4 (top) is a representative graph from one swine showing the returned difference signal at the four anatomical sites and the measured VGE.

![Fig. 2. Sensitivity for dual-frequency ultrasound (DFU) and B-mode ultrasound devices used in the study. (top) DFU was able to identify microbubbles statistically down to a concentration of 10 microbubbles/mL (\(* P < 0.01 \) compared with saline). (bottom) Using 6.6-MHz B-mode ultrasound microbubble sensitivity was \( 10^3 \) microbubbles/mL (\(* P < 0.05 \) compared with saline). Using 10-MHz B-mode ultrasound microbubble sensitivity was \( 10^2 \) microbubbles/mL (\(* P < 0.05 \) compared with saline). Error bars show +/- 1 standard deviation.

Figure 4 (bottom) shows only the femoral site from the same swine in the top graph.

The VGE data indicated that swine could be divided into two groups based on the amount of VGE measured postdecompression. One group, VGE High, contained swine with \( \text{bmdVGE} \geq 3 \) at any time point (\( n = 5 \)). The second group, VGE Low, contained swine with \( \text{bmdVGE} \leq 1 \) at all time points (\( n = 10 \)). Both the VGE High and VGE Low groups showed increases in microbubble signals above baseline (see Fig. 5), while the mock dive swine did not show any increase over baseline. The VGE High group (Fig. 5, left) showed an increase in difference signal above baseline immediately postive at \( t = 0, 5, \) and 10 min, and then the difference signal returned to baseline. This pattern suggests an initial rise in microbubbles, followed by a decrease. The VGE Absent group (Fig. 5, center) also showed an immediate increase in difference signal (\( t = 5 \) and 10 min) but difference signals were also significantly above baseline at
sensitivity in vivo is a function of both the target nonlinearity
and the system geometry (acoustic propagation paths and geometric
distribution of the microbubbles along the paths). It is not possible to say with
confidence that a measured difference signal level in one experiment (e.g., a swine) corresponds to the same micro-
bubble concentration measured in the sensitivity study.

Nevertheless, this level of sensitivity makes this technique suitable for detecting small concentrations of bubbles. In stud-
ies using B-mode ultrasound to identify microbubbles (e.g., to
detect wall motion abnormalities or vascular tumors), positive
identification relies on high concentrations of microbubbles.
The multiple bubbles within the ultrasound contrast agent
increases backscatter of the ultrasound and increases contrast
within the images. But, at lower concentrations, B-mode ultra-
sound is unable to differentiate small numbers of ultrasound
contrast bubbles from background reflectors. Our data show
that the DFU is capable of distinguishing microbubbles with
specificity at very low concentrations. The absence of a clear
microbubble return in the B-mode images is most likely due to
insufficient microbubble concentrations relative to other linear
scatter in the images.

Vascular detection of microbubbles. Our previous research
has shown that DFU can detect microbubbles injected into the
biceps femoris tissue sites and decompression-induced micro-
bubbles at tissue sites (biceps femoris) in swine (5, 21). The
current research investigated if DFU could be used to detect
decompression-induced microbubbles within the vascular sys-
tem. The data show that either the carotid, brachial, or femoral
sites can be used to detect vascular bubbles with DFU.

The time course of vascular detection showed a rapid rise
after bolus injection, followed by a decline. Microbubbles
detected after bolus injection were detectable for ~7 min. This
rate of decline matches the published half-life time (1) and is
similar to what is seen in clinical use of ultrasound contrast
agents (8). Injected microbubbles were detected at the three
measurement sites, with each site exhibiting the same response.
These vascular sites, carotid, brachial, and femoral, were chosen
for two reasons. One is that these vessels were large and
therefore we would be assured that the injected microbubbles
would be passing through them. Two, these vessels were
relatively superficial, lying anywhere from 1 to 4 cm deep,
falling into the measurement volume of our machine, which is
an approximate 5 mm³ cylinder centered 2.5 cm deep. These
results indicate that DFU is capable of detecting microbubbles
in vascular sites.

Microbubble detection following decompression. In swine
decompressed from 4.0 ATA, 2/8 had bmdVGE, while in the
4.5 ATA swine, 3/7 had bmdVGE. As in humans, there was
interworse variation in the development of bmdVGE (10). Following
decompression, microbubbles were detected at all
the vascular sites, as well as over the tissue site (i.e., biceps
tensoris). No site showed a greater propensity for microbubbles
over the others. These data suggest that DFU could be used at
a variety of sites on the body to detect microbubbles, including
tissue sites, which would be useful operationally in diving. We
are most likely detecting microbubbles between 1 and 4 μm in
diameter, as our ultrasound interrogation frequencies (2.25 and
5.00 MHz) are tuned for bubbles of that size (assuming they
respond similarly to free air bubbles in water). The environ-
ment surrounding the bubbles (e.g., the local fluid density and
pressure, location of the bubble relative to vessel walls, and the

DISCUSSION

The results of this study indicate that 1) the sensitivity of
DFU in vitro is on the order of 10 microbubbles/mL in water,
which may represent a physical limit; 2) DFU can be used at
several vascular sites to detect microbubbles in the circulation;
3) microbubbles are also detected at extravascular tissue sites;
4) microbubbles appear postdecompression regardless of the
VGE grade; and 5) an inverse relationship may exist between
microbubbles and bmdVGE.

DFU sensitivity study. The sensitivity of DFU, 10 micro-
bubbles/mL, was similar to theoretical limits as predicted by
the nonlinearities of microbubbles in water. In the same ex-
perimental facility, the microbubble concentration sensitivity
for the B-mode ultrasound machine used in our study was
found to be either 100X or 1000X less than DFU, depending
whether 6.6 MHz or 10 MHz transducer frequency, respec-
tively, was used. These results clearly show that DFU is more
sensitive to microbubbles than B-mode ultrasound in vitro.
The concentration of microbubbles in the bloodstream following
decompression is unknown, but the microbubble concentra-
tions likely start small and progress to higher levels. At these
smaller concentrations, DFU shows a clear advantage over
B-mode ultrasound in vitro. Whether the same concentration
sensitivity is achieved in swine depends on several factors. The
sensitivity in vivo is a function of both the target nonlinearity

later time points (t = 30 and 45). This pattern suggests a rise
in microbubbles that continued throughout the postdecompress-
tion time period. Taken together, these results suggest an
inverse relationship between microbubbles and VGE grade.
The VGE High group showed that as bmdVGE increased,
microbubbles decreased. Conversely, in the VGE Low group,
bmdVGE stayed low during the postdecompression period,
while microbubbles increased and remained elevated. The data
from the sham dive are plotted for comparison (Fig. 5, right).

Fig. 3. Detection of ultrasound contrast agent/microbubbles at three vascular
sites.
presence of any bubble shell) can influence their resonant frequency, thus altering the relationship between diameter and resonant frequency. Nevertheless, it is unlikely that these factors would alter the resonant characteristics dramatically from the normative values in water, and the bubbles detected by the DFU are likely less than 10 μm. At present, there is no independent method to ascertain this in vivo.

Our results show that microbubbles were present in swine that developed VGE and also in those that did not. Microbubbles increased immediately postdecompression in both groups of swine. In the VGE High group, microbubbles decreased over time, while the VGE grade increased. The reasons for the reduction in the microbubble signal are unclear. One reason may be that smaller microbubbles are the precursors to larger VGE. Over time postdecompression, the smaller microbubbles may decrease in number as they grow to become larger bmdVGE, resulting in the increase in VGE grade. The hypothesis that microbubbles are bmdVGE precursors has been discussed previously (3, 17, 22, 24) but the technology to prove this has been missing. The appearance of bmdVGE, and disappearance of microbubbles, may indicate that the balance of forces between bubble growth and bubble destruction (e.g., local nitrogen partial pressure, surface tension) has shifted toward bubble growth. As the bubbles grow or coalesce, they move out of the detectable range for the DFU, thereby reducing the returned DFU signal. This hypothesis is further supported by the results from the VGE Low group. This group showed increased microbubbles but little to no bmdVGE over the course of the entire postdecompression period. This suggests that there was no progression of smaller microbubbles to larger VGE. While these data do not prove that microbubbles grow or coalesce to form bmdVGE, these data support the hypothesis that bmdVGE begin as microbubbles.

A second explanation for the decrease in microbubble signal over time is that as VGE appear in the bloodstream, it is possible that they scatter the ultrasound signal and reduce the ultrasound incident on the microbubbles. The level of microbubbles may not actually change but only appear to change due to scattering of the ultrasound. An analysis of the returned frequencies (data not shown) do not show a diminution of either the pump or image frequency. Had the VGE been scattering the return signals, we would have expected a de-
The industrial transducers and data acquisition equipment used for DFU do not interfere with the diver’s work. Also, DFU is a low-power technique. The DFU transducers could be placed in locations at several body sites, which makes this a flexible monitoring technique. The DFU system itself is well-suited for real-time monitoring. The moving diver will provide its own set of challenges, the DFU research would be needed to demonstrate if this is the case. Further monitoring of bubbles is constantly changing, with new microbubbles being formed while other microbubbles are being destroyed or growing outside the detectable diameter range. But, to our knowledge, this is the first demonstration that there may be a population of circulating microbubbles that exist in the bloodstream prior to bmdVGE detection.

The data from this study strongly suggest that microbubbles precede bmdVGE and can be detected in the absence of bmdVGE. The bmdVGE have been linked to a variety of decompression-associated ailments (9, 12, 14–16, 20), and therefore an early detection method for decompression stress would be of great benefit. For example, if microbubbles appear during an ascent from a dive, the possibility exists that if the diver descended slightly or extended the time at a decompression stop, the microbubbles might disappear and serve as a marker that the ascent could continue safely. Further research would be needed to demonstrate if this is the case.

While the actual implementation of DFU device on a freely moving diver will provide its own set of challenges, the DFU technique itself is well-suited for real-time monitoring. The data from this study show that DFU could be used to monitor at several body sites, which makes this a flexible monitoring technique. The DFU transducers could be placed in locations that do not interfere with the diver’s work. Also, DFU is a low-power technique. The industrial transducers and data acquisition and processing equipment used in this study are well-suited for research, but the technique could be implemented using with small, flat transducers and low-power digital signal processors with the power supplied from a battery. Last, the DFU returns a difference signal at a frequency lower than the image frequency, which is likely to be attenuated less in transit.

**Limitations.** The main objective of this study was to determine if microbubbles could be detected in the bloodstream using DFU. The study was not designed as a head-to-head comparison study with a variety of other ultrasound techniques. So, we cannot rule out that under optimal experimental conditions other B-mode ultrasound and/or Doppler ultrasound devices might have detected a signal in the bloodstream earlier than the instrument we used. This needs to be tested in future studies. But, we believe this is unlikely since the concentration of the microbubbles we detected was most likely much less than the minimum concentration needed to see a bubble on B-mode ultrasound. Also, we compared the devices directly under ideal conditions [the mineral oil tank experiment (Fig. 2)] and showed a significant difference in sensitivity.

Our study used anesthetized swine, not active divers, so more work is needed to show if this technique could be applied to real world human applications. Physically active divers provide data acquisition problems that are more challenging than the instrument we used. While no research has shown an effect of our anesthetic on VGE production or decompression stress, this does not mean that one does not exist.

The data from this study do not allow for definitive conclusions about the attenuation of the microbubble signal once bmdVGE appeared. Microbubble detection clearly preceded bmdVGE detection in this study, but the reduction in the microbubble signal that was seen once bmdVGE appeared could be due to several factors.
Conclusion. Microbubbles appear in the bloodstream following decompression and can be detected at over both vascular and tissue sites using DFU. The microbubbles occur before bmdVGE and are inversely related to the appearance of bmdVGE. This temporal relationship supports the hypothesis that microbubbles are the precursors for larger bmdVGE. Microbubbles persisted in the absence of bmdVGE. The data also suggest that the DFU technique has potential as a method for early detection of decompression stress.

GRANTS
This work was supported by the Office of Naval Research (ONR N00014-02-1-0406).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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