Microvascular Permeabilization and Cardiomyocyte Injury Provoked by Myocardial Contrast Echocardiography in a Canine Model

Douglas L. Miller, PHiD,* Edward M. Driscoll,† Chunyan Dou, MD,* William F. Armstrong, MD,‡ Benedict R. Lucchesi, MD, PHiD†

Ann Arbor, Michigan

OBJECTIVES
The aim of this research was to evaluate the potential for myocardial contrast echocardiography (MCE) to provoke microscale bioeffects in a canine model.

BACKGROUND
Myocardial contrast echocardiography induces bioeffects in rat hearts, but translation of such results to larger animal models is uncertain.

METHODS
Dogs were anesthetized and prepared for open- (n = 1100522) or closed- (n = 6) chest MCE. Evans blue dye was injected intravenously as an indicator of microvascular leakage, and propidium iodide was used to stain for irreversibly injured myocytes in frozen sections. The contrast agent (Definity, Bristol-Myers Squibb Medical Imaging Inc., North Billerica, Massachusetts) was diluted in saline and infused intravenously at 2 l/kg/min. Myocardial contrast echocardiography in a short-axis (open-chest) or modified four-chamber view (closed-chest) with 1:4 end systolic electrocardiogram triggering was performed at 1.5 MHz for 10 min in a single imaging plane.

RESULTS
Petechiae and leakage of Evans blue were observed in the ultrasound scan plane within the anterior left ventricle. For 1.2 MPa and 2.2 MPa, open- or closed-chest MCE, Evans blue content in tissue within the scan plane was significantly greater than in tissue outside this plane. Counts of propidium-iodide-stained nuclei for 2.2 MPa open-chest MCE were also significantly greater inside than outside the scan plane.

CONCLUSIONS
In a canine model, MCE induces myocardial injury comparable to that seen in the rodent model. (J Am Coll Cardiol 2006;47:1464–8) © 2006 by the American College of Cardiology Foundation

Several intravenous (IV) ultrasound contrast agents have been approved in the U.S. and other countries, primarily for intracardiac cavity opacification (1). Many other agents and applications are being explored (2). All commercial agents consist of a suspension of stabilized microbubbles designed to interact strongly with diagnostic ultrasound, thus providing image enhancement. Although these agents appear to be relatively free of risk from a pharmacological viewpoint, the potential for adverse bioeffects of ultrasound contrast agents arises from the interaction of ultrasound imaging pulses and the stabilized microbubbles. This interaction is a form of acoustic cavitation, which is a well-recognized mechanism for nonthermal bioeffects of ultrasound (3).

For myocardial contrast echocardiography (MCE), microscale effects have been observed in isolated rabbit hearts (4) and in vivo in rats (5–7). Microvascular leakage induced with different agents was similar when compared on the basis of the number of stabilized microbubbles (5). Histologically defined microlesions have been identified by inflammatory cell infiltration in samples taken 24 h after MCE in rats (6), and vital staining has shown that cardiomyocytes are specifically involved within the microlesions (7). Previous studies in rodents have identified specific combinations of contrast dose, ultrasound power, delivery mode, and scanning duration below which bioeffects are avoided, and above which they are uniformly produced (5,7). Of note, while many of the doses and ultrasound powers used in previous work exceed what is currently employed clinically, they are within the allowable range for clinical work.

Translation of observations in rodents or ex-vivo preparations to larger animal models or to the clinical laboratory is often difficult. Uncertainty persists with respect to the relevance of microscale bioeffects seen in small animal models during MCE, owing to the differences in size and ultrasound delivery conditions. Premature ventricular contractions induced by MCE have been noted in rats (5) and in humans (8), but microvascular and cellular injury have not been demonstrated in a large-animal model. The purpose of the current study was to test the hypothesis that effects similar to those seen in rats can be produced in a canine model of MCE.
METHODS

Animal preparation. This investigation was conducted with the approval of the University Committee on the Use and Care of Animals, University of Michigan. A total of 28 dogs (25 female dogs) averaging 10.4 ± 1.5 kg in weight were anesthetized with an IV injection of 30 mg/kg pentobarbital. A cannula was inserted into the right jugular vein for injections, and electrocardiogram (ECG) electrodes were applied to three legs. For open-chest echocardiography in 22 dogs, the animal was intubated and ventilated. A left-lateral thoracotomy was performed to expose the heart, which was then suspended in a pericardial cradle. For six closed-chest dogs, the animal was anesthetized, the venous catheter inserted, and the ECG electrodes applied. The hair was carefully removed over the abdomen at the diaphragm. In 22 dogs (16 open- and 6 closed-chest), Evans blue dye in saline was injected intravenously as a tracer for microvascular leakage at a dose of 50 mg/kg 5 min before MCE. In 6 open-chest dogs, propidium iodide (PI) (1 mg/kg) was slowly infused into the left atrium approximately 5 min after the cessation of MCE, as a vital stain for irreversibly injured cells (9).

Ultrasound contrast agent. A new vial of Definity (Bristol-Myers Squibb Medical Imaging Inc., North Billerica, Massachusetts) was prepared for each dog according to the manufacturer's instructions. For infusion, the agent first was diluted to 20 μl/ml with sterile saline in a 20-ml syringe. The syringe was then mounted in a syringe pump (Model 11 plus, Harvard Apparatus, Holliston, Massachusetts) set to deliver 0.1 ml/kg/min. The jugular vein catheter was connected directly to the syringe. The suspension was infused for 2 min before MCE, then for 10 min of MCE. Thus, the agent dose rate was 2 μl/kg/min with a total dose of 24 μl/kg (20 μl/kg during echocardiography).

Ultrasound. An unmodified commercially available diagnostic ultrasound platform was used for all imaging (GE Vingmed System V, General Electric Co., Cincinnati, Ohio) using a cardiac scanhead (FPA2.5). For the open-chest dogs, an ultrasound probe cover was placed loosely over the probe, which was aimed vertically downward. The end of the probe cover was filled with degassed saline and used as a flexible 4-cm stand-off from the heart surface. This provided a clear short-axis view of the heart at the level of the papillary muscles. For the closed-chest dogs, the probe was coated with ultrasound coupling gel and pressed onto the abdomen and aimed horizontally at the heart using a ring-stand arrangement. Transthoracic views, as used clinically, were not possible in the small dogs, because the anatomical arrangement of lung tissue blocked the sector scan. The transabdominal view included attenuation by 3 to 4 cm of intervening tissue, and also was partly restricted by the lungs. Initial real-time imaging at a transmit frequency of 1.5 MHz with a 36.4 Hz frame rate, 10 cm depth, and 5 cm focus was used to aim the probe.

The peak rarefractional pressure amplitude (peak RPA) produced by the phased array system was measured in a water bath, as described previously (5). The peak RPA was adjusted by the power control to provide either −2.2 MPa or −1.2 MPa. These correspond to equivalent mechanical index (MI) values (the peak RPA divided by the square root of the frequency) of 1.8 and 1.0, respectively (i.e., less than the upper limit of 1.9 for diagnostic ultrasound). The RPA within the myocardium was nonuniform with depth because of the beam geometry for the 5-cm focal zone with the maximum RPAs arranged to cover the anterior left ventricular (LV) wall. For open-chest studies, single-image frames were triggered from the ECG signal each four beats at the end of systole. For the closed-chest model, attenuation reduced the RPA values. Assuming a nominal attenuation of 0.5 dB/cm/MHz (3) over 4.0 cm between the probe and heart surface, the peak RPA was reduced to 1.6 MPa (equivalent MI = 1.3). Dual frames, 33 ms apart, were triggered at four-beat intervals. For sham MCE, echocardiography was performed for 10 min, followed by injection of the contrast agent with the ultrasound off. The MCE images together with ECG were recorded on videotape for later analysis.

Experimental plan and statistical analysis. For the open-chest model, samples inside and outside the scan plane were evaluated for microvascular leakage in five hearts each for the sham, 1.2 MPa, and 2.2 MPa conditions, and for PI staining in three hearts with the MCE scan plane above the sham MCE scan plane and three with the reverse placement. Six closed-chest MCE hearts were evaluated for microvascular leakage. Numerical results are presented as the mean ± SD or plotted as the mean with standard error bars. For statistical analysis (Sigmastat 3.1, Systat Software Inc., Point Richmond, California), paired Student t tests or Mann-Whitney rank sum (MWRS) tests (for data that failed tests for normalcy and equal variance) were used, with statistical significance assumed at p < 0.05.

Measured end points. Animals were euthanized 15 min after scanning by IV injection of a lethal dose of potassium chloride, after which the heart was removed and perfused with 120 ml of heparinized saline. The exposure scan plane was identified by a blue band formed by leakage of the dye from the microvasculature. Samples of myocardium were obtained from five myocardial strips, each 1-cm wide, cut perpendicular to the blue band on the anterior LV as indicated in Figure 1. For each strip, a sample was taken from within the blue band and another from a position

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<th>Abbreviations and Acronyms</th>
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<tr>
<td>IV = intravenous</td>
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<td>LV = left ventricle/ventricular</td>
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<td>MCE = myocardial contrast echocardiography</td>
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<td>MI = mechanical index</td>
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<td>MWRS = Mann-Whitney rank sum</td>
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<td>PI = propidium iodide</td>
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<td>RPA = rarefractional pressure amplitude</td>
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1cm distant, which was out of the plane of exposure. The dye was then extracted from the samples with formamide, and quantified using a spectrophotometer as described previously (5). For sham exposure, the positions of the samples were selected to mimic those of the MCE samples. For the closed-chest model, two strips were cut at the center and the results combined to give one result comparable to the center strip cut for the open-chest model.

Cardiomyocyte injury was assessed using PI as a stain for nuclei of irreversibly injured cells (9). Samples taken for histology after MCE showed contraction band necrosis and petechial hemorrhage, as for the rat heart study (6), but these effects were not quantifiable. For the PI experiment, a slightly different open-chest scanning procedure was used in order to combine sham and exposed conditions in each test. Ten minutes of echocardiography without contrast agent (sham MCE) was completed, the probe was moved 2 cm, and 10 min of MCE was completed at the new position. The sham MCE and MCE scan planes were alternately 1 cm above or below the scan position at the papillary muscle used for the open-chest microvascular leakage tests. Tissue samples were frozen, and 10-μm sections were mounted for examination under a fluorescence microscope.

RESULTS

Open-chest MCE at 2.2 MPa produced microvascular leakage across the scan plane in all instances as shown in Figure 1. The blue band formed across the left ventricle at the ultrasound scan plane and records the microvascular leakage, with irregular erythrocyte extravasation in the center of the band (arrows). The vertical lines indicate the orientation of tissue slices used for samples A to E. Scale bar = 1 cm.

Figure 1. Photograph of a heart after myocardial contrast echocardiography in the open-chest model at 2.2 MPa. The blue band formed across the left ventricle at the ultrasound scan plane and records the microvascular leakage, with irregular erythrocyte extravasation in the center of the band (arrows). The vertical lines indicate the orientation of tissue slices used for samples A to E. Scale bar = 1 cm.

Asation extended through the thickness of the anterior LV wall, as shown in Figure 2. The dye content was essentially the same for all five sample positions (p = NS). A blue band was evident for the 1.2-MPa samples. Neither petechiae nor Evans blue leakage was evident for the sham samples. The mean results for the center samples of each heart are shown in Figure 3. In paired t tests, all the results were significantly different inside and outside the blue band (Fig. 3). In unpaired t tests, the closed-chest (p < 0.02) and 2.2 MPa open-chest (p < 0.001) results from inside the band were significantly greater than the sham. The blue bands for the closed-chest MCE hearts were not as well defined for the open-chest samples, as shown in Figure 4. Results, analyzed in two samples each inside and outside the blue band, are presented in Figure 3 for the six hearts (using averages of the two samples).

Six hearts were scored for PI staining; PI-stained nuclei were present around the periphery of sections, and red autofluorescence in arterioles in the interior of samples was present in both sham and MCE. However, there was consistent enhancement of the number of red fluorescent spots seen within the MCE band relative to the other samples. Red fluorescent spots seen under low magnification proved to contain clusters of several fluorescent nuclei when examined under high magnification. It was not possible to determine if these were cardiomyocyte or endothelial cells. For counting the number of red fluorescent spots, four photonmicrographs (each covering 1.56 mm²) in red fluorescence were taken in the interior of a section and enhanced in contrast. The processed images were scored blind. The scores for the four frozen section photographs from each of three positions in six dogs were 2.3 ± 2.0/mm² outside and 27.5 ± 16.5/mm² (MWRS test, p = 0.002) inside the scan plane.

Figure 2. A photograph of a slice of the left ventricle cutting across the scan plane from the heart shown in Figure 1. The cut surface of the anterior wall is about 1-cm wide (two-headed arrow) and in focus with the interior surface of the left ventricle falling out of focus to the right. The petechiae and Evans blue leakage penetrate through the anterior wall, and a blue band can be seen on the interior left ventricular surface. Scale bar = 5 mm.
Premature complexes were also observed and counted in the ECG record. No premature complexes were evident for either the sham, 1.2 MPa open-chest, or closed-chest MCE. For the open-chest 2.2 MPa microvascular leakage tests, premature complexes averaged 120 per 1000 for the PI tests, the position was changed with MCE approximately 1 cm above the previous position for three dogs and 1 cm below for three dogs. The premature complexes were 19 for above and below, respectively, or 12 combined. The combined premature complex count for the PI tests was significantly less than the count for the microvascular leakage tests (MWRS test, p < 0.005), which suggests some influence of scan position.

DISCUSSION

These results, reported in a canine model of MCE, closely parallel the results previously reported from our laboratory using a rat model of MCE. Both microvascular leakage and cellular injury were demonstrated for high MI-triggered imaging. Our previous work was designed to delineate the range of bioeffects that occur and has identified the ranges of ultrasound power, delivery mode, and contrast agent dose below which adverse bioeffects are minimized and above which bioeffects are uniformly produced (5,7).

Myocardial contrast echocardiography is a field in evolution for which multiple imaging algorithms have been proposed, including low MI continuous imaging, intermittent triggered high MI imaging, and burst imaging followed by low MI imaging. Our previous work suggested that low MI imaging at a low dose of contrast agent is unlikely to result in bioeffects. Current MCE protocols typically utilize low MI imaging with only intermittent high MI bursts, and it is unlikely that this more conservative algorithm would result in a similar degree of bioeffects as noted here. However, higher dose intermittent triggered imaging has the potential to result in bioeffects, especially if combined with higher contrast agent doses or prolonged scanning times. While many of the imaging algorithms utilized in our animal work exceeded the contrast dose, or ultrasound exposure time currently used in clinical practice, they are well within the range of commercially available clinical ultrasound platforms, and the lower doses resulting in adverse bioeffects have been within the range of recommended doses.

Anecdotally, previous investigators have not noted similar levels of capillary leakage or histologic disruption in numerous studies evaluating either the physiologic behavior of MCE or its diagnostic utility in open- and closed-chest large animal models. The reason for the absence of similar bioeffects in the previous studies is most likely that Evans blue dye was not used, and the hearts were not removed and perfused to increase the contrast between the area of microvascular damage and the adjacent normal tissue. Furthermore, the majority of MCE studies performed either in the animal laboratory or clinically involved examination of multiple different scan planes rather than continuous prolonged imaging along a single scan plane. This has the effect of “diluting out” the total ultrasound delivery to the myocardium and would be expected to result in substantially less obvious evidence of bioeffects.

Study limitations. There are limitations to the research reported here. First, the data obtained in a 10-kg canine may not translate with respect to ultrasound delivery to a normal-

Figure 3. Results for microvascular leakage for sham exposure and open-chest myocardial contrast echocardiography at 1.2 MPa, 2.2 MPa, and closed-chest myocardial contrast echocardiography. Samples were obtained from inside and outside the blue band (or at the same positions for the shams). The p values are for paired t tests comparing the inside and outside measurements.

Figure 4. A photograph of the blue band (arrows) generated by myocardial contrast echocardiography in the closed-chest model.
sized adult. However, the overall scan depth and both theoretic and measured attenuation suggests that the more proximal cardiac structures in a normal-sized adult would be subject to similar levels of ultrasound delivery using identical protocols. Secondly, in this study we evaluated only one contrast agent. We have previously demonstrated equivalent bioeffects with multiple contrast agents that are quite similar in magnitude after correction for the number of microbubbles present in a given volume of the agent (5). Finally, this study involved evaluation of only one commercially available ultrasound platform. Whether similar bioeffects would be seen using other ultrasound platforms remains conjectural.

**Conclusions.** Bioeffects including evidence of capillary leakage and irreversible tissue injury were identified in a canine model of MCE using contrast doses and diagnostic ultrasound delivery modes optimized for induction of adverse bioeffects. These observations confirmed that bioeffects comparable to those seen in rats can occur in the larger animal model, and have direct relevance for avoiding bioeffects in the clinical setting.

**REFERENCES**