Histological Characterization of Microlesions Induced by Myocardial Contrast Echocardiography

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Background: Myocardial contrast echocardiography (MCE) has been shown to have a potential for apparently reversible side effects related to the interaction of ultrasound with the contrast microbubbles, including premature ventricular contractions and microvascular leakage. We investigated the potential for high-dose MCE to induce histologically definable microlesions. Methods: Myocardial contrast echocardiography with 1:4 end-systolic triggering was performed at 1.5 MHz and 1.7 mechanical index in a short axis view of the left ventricle in rats. Two high doses (500 μl/kg) of Optison® agent were given 5 minutes apart during 10 minutes of echocardiography. For histology, the hearts were perfused and fixed in 10% neutral-buffered formalin. Slides from rats sacrificed 1 day after MCE were scored blind by a pathologist, and, in addition, photomicrographs in the anterior half were evaluated by digital image analysis. Results: In rats sacrificed 10 minutes after MCE, microvascular leakage and petechiae were highly significant. However, lesions displaying necrotic debris associated with inflammatory infiltrates were not histologically evident at this time. Heart samples 24 hours after MCE showed microlesions with inflammatory infiltrates scattered primarily over the anterior half of the sections. Pathologically, there was inflammatory cell infiltration in areas of 0.6 ± 0.5% for shams and 3.6 ± 3.6% for MCE ($P < 0.01$). Analysis of photographs from the anterior wall found microlesion areas of 0.5 ± 0.8% for shams and 7.4 ± 5.0% for MCE ($P < 0.02$). For rats sacrificed 1 week and 6 weeks after MCE, the microlesions healed to form small fibrous regions interspersed with normal myocytes. Conclusion: High-dose MCE has a potential for causing microscale lesions in the myocardium and the possibility of therapeutic applications. (ECHOCARDIOGRAPHY, Volume 22, January 2005)

ultrasound contrast agent adverse effects, *Optison®*, *capillary leakage*, *acoustic cavitation*, *premature ventricular contraction*, *cardiomyocyte necrosis*

Several commercial ultrasound contrast agents are approved in the United States for the clinical application of left ventricular opacification, and have been shown to be safe and efficacious within the clinical approval process. Others agents and applications to other tissues are under development and evaluation.[1,2] Ultrasound contrast agents are distinctly different in their characteristics, but all commercial agents depend on the echogenicity of gas bodies (stabilized gas-filled microbubbles) for the contrast effect. The interaction between ultrasound pulses and the gas bodies is a form of acoustic cavitation, a well-known mechanism for bioeffects.[3] Bioeffects specifically related to ultrasound contrast agents have been the subject of several recent reviews.[3–6] The interaction produces pulsation, which may involve destabilization and destruction of the gas bodies, and induction of mechanical perturbations in their immediate vicinity. Since gas bodies or other cavitation nuclei are normally scarce in vivo, this perturbation represents a new physical means of biological intervention, which has qualitatively different characteristics from ultrasonic heating or other physical modalities.

In vivo capillary rupture and petechial hemorrhages can be induced by diagnostic ultrasound scanning of skeletal muscle with

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contrast agents in the circulation.[7–9] More recently, research on myocardial contrast echocardiography (MCE) using diagnostic ultrasound scanners has demonstrated potential side effects such as premature ventricular contractions (PVCs) in humans,[10] erythrocyte extravasation in isolated rabbit hearts,[11] mild troponin T elevation but no pathohistological damage after rat heart scanning,[12] and capillary leakage together with PVCs and petechiae in rat hearts.[13] The microvascular leakage induced during MCE with different agents appears similar when compared on the basis of gas body dose.[14] The effects appear to be mostly reversible; for example, PVCs end immediately and the microvascular leakage ends within 20 minutes after cessation of MCE.[15] Several recent studies have suggested a potential therapeutic role for purposeful microvascular damage induced by gas body destruction for either drug or gene vector delivery using diagnostic ultrasound.[16–20] Nondiagnostic ultrasound at relatively high-pressure amplitudes with contrast agents caused arrhythmias and histologically defined myocardial degeneration in rat hearts.[21] Gas body enhanced ultrasound has been proposed as a means of surgical intervention[22] and of simultaneous tumor ablation and gene transfer in cancer treatment.[23]

The overlap between diagnostic and therapeutic applications of MCE is presently unclear, particularly with regard to the potential for irreversible, histologically definable damage. The purpose of this study was to investigate the possibility that histologically definable microlesions might be created, in association with the previously identified petechiae, in a well-defined rat model of MCE. Relatively high agent doses of Optison and high-power settings were used to elicit any possible histologically observable bioeffects, and for following the time course of microlesion healing. The results establish a potential for microscale lesions from diagnostic ultrasound with high contrast dosage and gas body destruction, which presents a new tool for medical research and therapeutic applications.

Methods

Animal Preparation

All in vivo animal procedures were conducted with the approval and guidance of the University Committee on Use and Care of Animals. Briefly, a total of 37 CD hairless rats (Charles River) were anesthetized by intraperitoneal injection of a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg). A 24-gauge cannula was inserted into a tail vein for injections. Waterproof electrodes (LL911, Lead-Lock, Inc., Sandpoint ID) were applied to three legs for ECG acquisition. The rats were each mounted in a 37 °C degassed water bath with its head supported above the water. In 10 rats, Evans blue dye in saline was used at a dose of 50 mg/kg as a marker of microvascular leakage. The methods have been described in further detail previously.[13]

Ultrasound Contrast Agent

The commercial ultrasound contrast agent Optison® (Amersham Health Inc., Princeton NJ) was resuspended by inverting and rotating the vial until the suspension was milky white. This agent contains approximately $5–8 \times 10^8$ per ml gas bodies 2–4.5 μm in diameter, which are perfluoropropane filled and stabilized by an albumin shell. A reservoir of perfluoropropane was connected to the dispensing-pin vent to maintain the gas in the head space of the vial, and a fresh vial was used each day. Two doses of 500 μl/kg were given as 10 s bolus injections 5 minutes apart during 10 minutes of MCE. For comparison, this is about 140 times the recommended and 14 times the maximum doses of 0.5 ml and 5 ml noted in the package insert for an adult human (assuming 70 kg).

Ultrasound

A commercial diagnostic ultrasound machine (GE Vingmed System V, General Electric, Cincinnati OH) was employed as the ultrasound source. A cardiac phased array ultrasound probe (FPA2.5) was clamped in the water bath and used to acquire a left ventricular short-axis view of the heart in the intact rat at a distance of 4–5 cm from the transducer face. This ultrasound scanning setup allowed essentially free field exposure conditions and placement of the rat heart at a focal depth (5 cm) similar to human scanning. An initial image was obtained at high frequencies, then switched to 1.5 MHz for MCE with a 60 Hz frame rate. The maximum pulse during each scan had a peak rarefactional pressure amplitude (PRPA) of −2.3 MPa and a duration of 1.45 μs, measured in the water bath in the absence of the rat as described previously.[13] After adjustment for the estimated attenuation of 12% (−1.2 dB) through the rat chest wall, the in situ PRPA was

2.0 MPa. This corresponds to a mechanical index of 1.7, which is within the guideline limit for diagnostic ultrasound (MI < 1.9). The ECG was used to trigger frames at end-systole every fourth beat to allow refill of the capillaries with fresh gas bodies (continuous scanning destroys gas bodies in the ventricles and reduces capillary effects). The two sham conditions of ultrasound alone and contrast agent alone were combined. That is, sham treatment consisted of echocardiography for 10 minutes, followed by injection of the contrast agent with the ultrasound scanhead aimed away from the rat. All echo images together with ECG traces were recorded on SVHS video tape for later analysis.

Experimental Plan

Histological examination was performed on 27 rat hearts. Rats were anesthetized and sacrificed by bilateral pneumothorax. The hearts were excised and flushed with 10% neutral buffered formalin, and then stored in the fixative. Paraffin imbedding and histological sectioning were performed in the Histology Research Laboratory (University of Michigan Pathology Department). Preliminary studies demonstrated that microlesions could be identified in the rat hearts 1 day after MCE, and this was chosen as the primary time point with five sham and five MCE rats. In order to follow the time course of the microlesion healing, rat hearts were also examined at 10 minutes after sham or MCE (3 rats each), 1 week after MCE (5 rats) and 6 weeks after MCE (one control and 5 MCE rats). All sections were stained with hematoxylin and eosin, and alternate sections of the 6-week group received trichrome staining. For the 1 day post-MCE group, the slides were evaluated blind by a pathologist with visual estimates generated of the percentage area of the sections affected by microlesions with inflammatory cell infiltration, by necrosis, and by hemorrhage. It was noted that only the anterior half of the hearts had consistent evidence of microlesions. This distribution was likely due to the use of relatively high dose contrast, which causes shadowing of the posterior regions and hence protects the more distal structures from bioeffects of the ultrasound beam. For additional evaluation, the anterior half of the heart sections were photographed at 20× magnification at seven fixed positions and analyzed to determine the area occupied by microlesions. For this determination, photographs made in a bright field were added to red fluorescence images. This emphasized the darker microlesion areas, which had less fluorescent eosin staining than the normal tissue. The microlesion regions were measured using image analysis software (SigmaScan 5.0, SPSS Inc. Chicago IL) to outline the regions compared to the total area of the sample (excluding blood vessels, ventricular space or exterior space). For the other groups, areas of tissue were examined to find indications of the microlesion areas. These could not be clearly quantified and were qualitatively compared to the 1 day post-MCE group.

The exposure procedure differed from our previously described protocol for a rat model of MCE (i.e., 1.7 MHz with one Optison dose[13]). For comparison to the earlier work, an additional five sham and five MCE rats were handled and evaluated using the previous protocol, except using the new exposure procedure. These rats had an IV injection of Evans blue dye at 50 mg/kg before exposure, and were sacrificed 5 minutes after MCE. Data were gathered on PVCs, petechiae, and Evans blue dye contents as described previously.[13]

Numerical results are presented as the mean ± standard deviation, or plotted as the mean with standard error bars, for five measurements in different rats. For statistical analysis, Student's *t*-tests or the Mann-Whitney rank sum test, as appropriate, were used to compare means of the measured parameters, with statistical significance assumed at $P < 0.05$.

Results

In hearts obtained 10 minutes after MCE, there was a strong response in terms of PVCs, petechiae and Evans blue leakage. Results are given in Table I for five sham and five treated rats. The PVC count was about three times

TABLE I

Results for PVCs, Petechiae, and Microvascular Leakage of Evans Blue Dye for Five Sham and Five MCE Rats given as the Mean Values ± One Standard Deviation with the P-Value for the Student's *t*-Test Comparing Sham and MCE Results

	Sham	MCE	P
PVCs count	0 ± 0	124 ± 37	<0.001
Petechiae count	2.4 ± 2.3	290 ± 77	<0.001
Evans blue content (μg/g)	12.8 ± 2.2	78.3 ± 13.2	<0.001

higher, the petechiae count was about twice as high and the Evans blue content was about three times greater than for the previous exposure procedure.[13] In three treated hearts obtained for histology (without Evans blue), the scanned region was readily identifiable by the large number of petechial hemorrhages and overall red coloration as shown in Figure 1A. In histological sections, leakage of erythrocytes into the interstitium had clearly occurred in some regions as shown in Figure 1B. The layer of erythrocytes adjacent to the epicardial surface presumably is an example of the externally visible petechiae counted on the surface. The erythrocyte extravasation was not visually quantifiable relative to three shams (Fig. 2A), because erythrocytes often remained present within both sham and MCE sections after flushing of the hearts, and could not be clearly classified as extravasated or not. Furthermore, many myocytes appeared to be damaged, as evidenced by contraction band necrosis, but these specific areas of muscle damage were difficult to define or quantify (see Fig. 2B). Lesions displaying necrotic debris associated with inflammatory infiltrates were not histologically evident at this time.

In hearts obtained for histology 1 day after MCE, microlesions were identified by inflammatory cells, presumably macrophages, which occupied regions approximately the expected size of a few cardiomyocytes. The sham heart samples showed no or extremely minimal indication of such lesions. The pathologic results for the entire sections are plotted in Figure 3. These results revealed inflammatory response areas of $0.6 \pm 0.5\%$ for shams and $3.6 \pm 3.6\%$ for MCE ($P < 0.01$ using the Mann-Whitney rank sum test of significance), necrotic response areas of $0.6 \pm 0.5\%$ for shams and $1.4 \pm 0.5\%$ for MCE (not significant), and no quantifiable evidence of interstitial hemorrhage. The MCE heart samples showed microlesions scattered primarily over the anterior half of the sections. The digital image analysis of the photographs in the anterior half of the sections revealed an average count of 8.4 ± 6.2 and 0.4 ± 1.7 ($P < 0.001$) microlesions in MCE and sham sections, respectively. These occupied areas of $0.5 \pm 0.8\%$ for shams and $7.4 \pm 5.0\%$ for MCE ($P < 0.02$). This result is plotted in Figure 3, and is about twice the area percentage seen for the entire sections, as expected. The results were somewhat variable, which may be due to the variable interposition of lung tissue, ribs, or sternum between the scanhead and the heart, which resulted in low or no effects at some positions. For the anterior portion of the septum, the production of microlesions was relatively reliable, with an average count of 14.0 ± 4.5 microlesions occupying $10.8\% \pm 3.3\%$ of the muscle area ($P < 0.001$ relative to zero microlesion area in 5 shams at this position).

The appearance of the microlesions progressed over a 6-week period. Examples are shown in Figure 2 for a sham sample and for samples obtained 10 minutes, 1 day, 1 week, and 6 weeks after MCE. By the 6th week, the lesions appear as collapsed cell-free spaces on hematoxylin and eosin staining. Trichrome staining indicated minimal fibrosis in these lesion areas, with deposition of collagenous fiber, indicative of microscar formation. The healing process therefore appeared to follow a normal course of damage, inflammatory cell infiltration, and scar formation process with shrinkage of the tissue.

Discussion and Conclusions

In this study, high-dose MCE induced histologically definable lesions at high MI values. This effect appears similar to, though less severe than the diffuse effects seen at very high pressure amplitudes.[21] It is uncertain why histological evidence of damage was not seen previously in apparently similar examination of MCE-exposed rat hearts.[12] The effects were seen in this study at somewhat higher doses, which were delivered by bolus injection (rather than infusion), at higher MI values and were judged by different criteria, than for the reported negative pathohistological results,[12] all of which might be important factors. The myocyte loss apparently is related to reported endothelial damage[11] and petechia,[13] even though the microvascular leakage is rapidly reversible.[15]

Commercial ultrasound contrast agents, such as Optison®, have been tested for clinical safety and efficacy for diagnostic applications. However, a potential for microscale bioeffects exists with these agents, if used with high MI ultrasound exposure. In this study, evidence of microscale damage was sought by histological examination of rat heart samples obtained after very high dose, high MI MCE. Previous work has indicated proportionality of effects such as petechiae to the agent dose at low doses, but a leveling off of effects at high doses as used in this study.[13,14] Thus, it is difficult to extrapolate these observations to potential effects at clinical

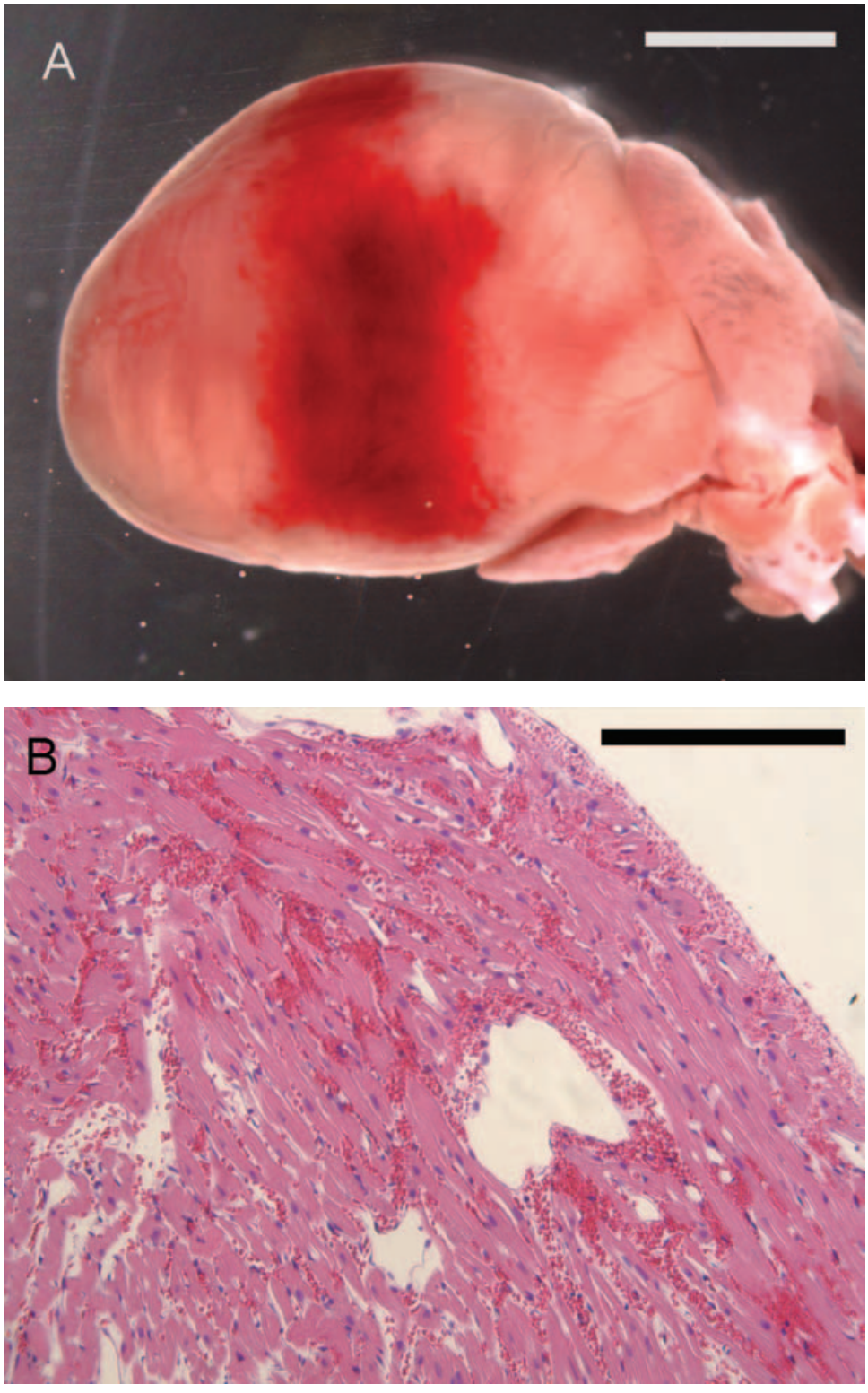

Figure 1. *Photograph of a rat heart 10 minutes after MCE, excision and flushing (***A***, scale bar 5 mm) and of a histological section in the scanned region (***B***, scale bar 200 μm). The reddish appearance of the scanned plane in (***A***) results from the many extravasated erythrocytes evident in (***B***).*

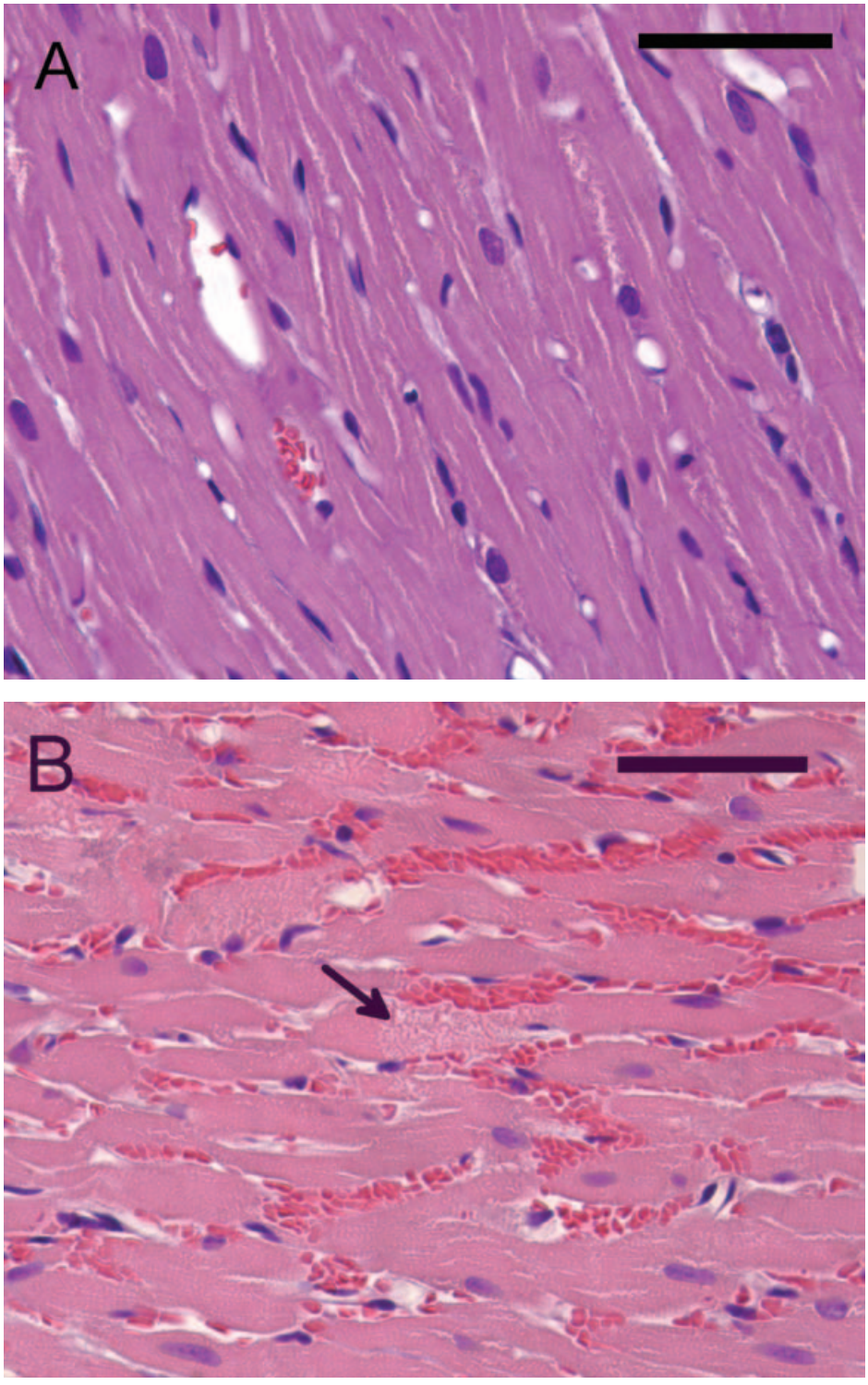

Figure 2. *Photomicrographs of heart sections taken at various times after MCE: (***A***) sham, and (***B***) 10 minutes, (***C***) 1 day, (***D***) 1 week, and (***E, F***) 6 weeks after MCE. All these images were taken at approximately the same position in the anterior left ventricular wall near the septum. The arrow in (***B***) indicates a cell with contraction band necrosis. The trichrome stained section (***F***) was 6 weeks after MCE, and adjacent to (***E***), with collagenous fiber stained blue. Scale bars are 50 μm long.*

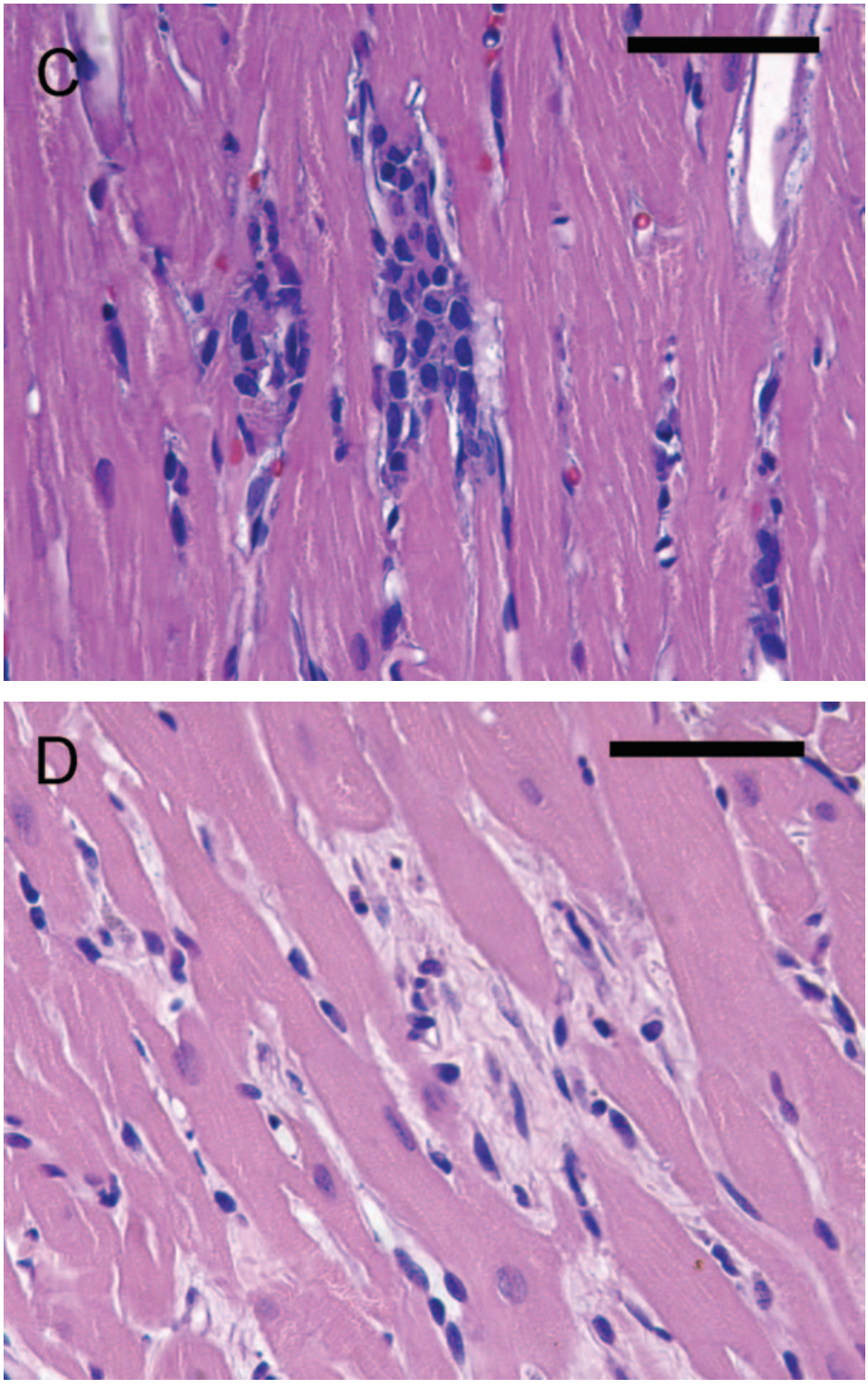

Figure 2. *Continued.*

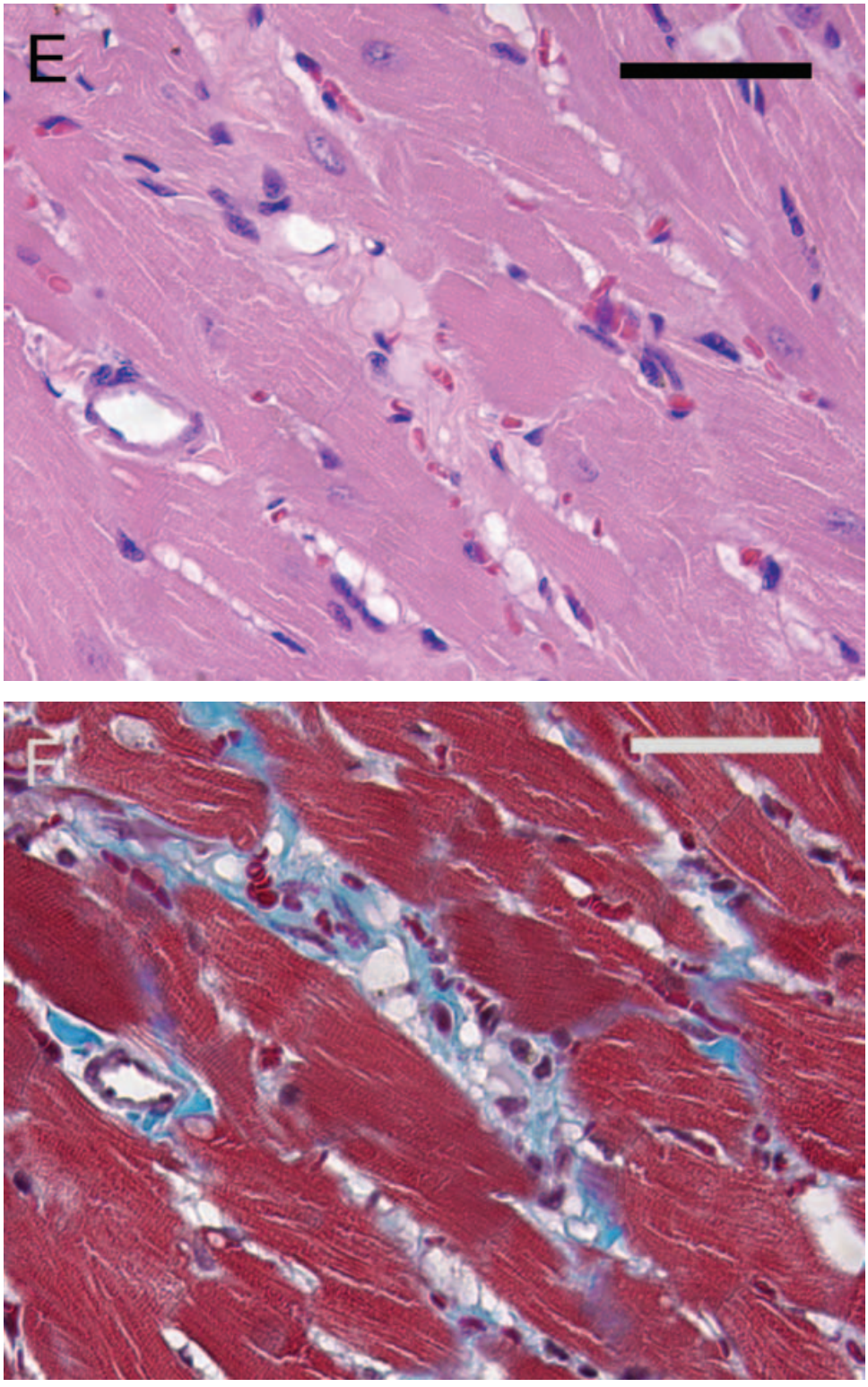

Figure 2. *Continued.*

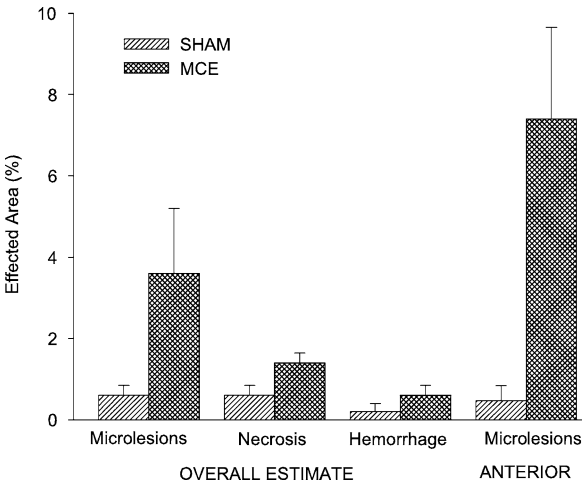

Figure 3. *The percentage area of heart tissue affected by lesions with inflammatory cell infiltration, by necrosis and by hemorrhage, as judged by a pathologist, and the area of microlesions measured from photographs of the anterior half of the hearts.*

diagnostic doses and MI values. Further study is needed to clarify the parameter boundary between diagnostic and therapeutic applications of gas body enhanced ultrasound. The results in this study do not contradict the previous findings of safety and efficacy; however, the lowest efficacious MI and contrast dosages should be used to maximize the safety profile of MCE.

For high agent dosage and high MI scanning, up to 10.8% of the myocardial area in anterior regions of histological sections was involved in microlesions. The microlesions represent cardiomyocyte loss and, as followed through a 6-week healing process, appear to result in fibrous microscar formation with tissue shrinkage. The microlesions were randomly distributed in the anterior wall, but the posterior wall was spared due to shadowing from contrast agent in the ventricles. No large continuous areas of destruction or infarct was seen. This type of diffuse tissue ablation may be suitable for therapeutic myocardial mass reduction, such as in hypertrophic cardiomyopathy. For patients unresponsive to medical therapy or cardiac pacing, intervention is required to reduce myocardial septal mass in an effort to reduce left ventricular outflow tract obstruction. Currently, myocardial mass reduction is accomplished by surgical myectomy[24] or alcohol ablation.[25,26] Several advantages might exist for gas body enhanced ultrasound over these other methods for myocardial reduction. The method would be noninvasive and utilize concomitant realtime echocardiography for precise guidance and aim of the ultrasound beam. In theory, it may be possible to selectively create areas of myocardial mass reduction in targeted areas without gross tissue structure disruption or infarction.

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