Ultrasonic backscatter and collagen in normal ventricular myocardium

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ABSTRACT Integrated ultrasonic backscatter has been related to collagen deposition in fibrotic myocardium. The purpose of our study was to measure the integrated ultrasonic backscatter in the right and left ventricles of 10 normal freshly excised canine hearts and five normal formalin-fixed human hearts. A 2.25 MHz, 50% fractional bandwidth transducer was positioned at the transducer focal distance from the epicardium. The radio frequency backscatter signal, excluding specular reflections, was digitized, squared, and integrated to yield the integrated ultrasonic backscatter (in decibels down from a 100% reflector). The segment of myocardium corresponding to the integrated ultrasonic backscatter sample volume was excised and assayed for hydroxyproline, a marker for collagen. A second purpose of our study was to evaluate the influence of fixation with formalin on the backscatter. Regional integrated ultrasonic backscatter was therefore measured in 10 freshly excised canine left ventricles, which were fixed in 10% formalin for 2 weeks. Integrated ultrasonic backscatter measurements were then repeated. In freshly excised canine hearts, the integrated ultrasonic backscatter from right ventricle was higher than that from left ventricle (−60.4 ± 1.6 [SEM] vs −66.9 ± 1.0 dB; p < .001). The collagen content of right ventricle was also higher than that of left ventricle (4.40 ± 0.26 [SEM] vs 3.58 ± 0.13 μg/mg dry weight; p < .005). Similar results were obtained in human hearts. There were no correlations between integrated ultrasonic backscatter and collagen content (r = .28 and .32 for dogs and humans, respectively). The integrated ultrasonic backscatter in freshly excised canine left ventricles was −64.8 ± 0.7 dB, which was not significantly different than the value of −65.6 ± 0.7 dB measured after fixation with formalin. We conclude that both integrated ultrasonic backscatter levels and collagen content are higher in the right ventricle than in the left ventricle of normal hearts. Formalin is a suitable fixative for ultrasound studies of the myocardium in vitro.

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BOTH the importance and limitations of M mode and two-dimensional echocardiography are well known to clinicians. Current clinical applications of echocardiography depend on specular reflection occurring at tissue interfaces to depict such structures as the epicardium, endocardium, and valve leaflets; standard echocardiographic examinations are not designed to evaluate the myocardium itself. This limitation has prompted interest in other acoustic variables of potential clinical relevance for characterization of structural changes in the myocardium. These include ultrasound absorption, attenuation, acoustic impedance, backscatter, and velocity in soft tissue. The utility of such parameters for noninvasive clinical diagnosis awaits the demonstration of a specific and reproducible relationship between one (or more) of these ultrasonic parameters and normal and pathologic states of the myocardium, as well as the technical capability to obtain these data in patients.

Among these variables, ultrasonic backscatter shows the most promise for clinical application by means of pulse-echo instrumentation. Backscatter may be described as low-amplitude, phase-sensitive echoes arising from within soft tissue, which are redirected back to the ultrasonic transducer. Rigorous definitions and methods for quantitation of tissue backscatter in the time and frequency domains have been given by various authors.

Although not all of the determinants of backscatter from myocardium are understood, results of previous experimental studies by Mimbs et al. have indicat-
ed that collagen may be an important determinant of backscatter. These investigators found significantly increased backscatter in the hearts of rabbits and dogs 5 to 16 weeks after infarction and in the hearts of rabbit after cardiotoxic doses of doxorubicin. In both lesions these changes were associated with an increase in the collagen content based on assay of hydroxyproline. In the doxorubicin study, these authors suggested that increases in the 5 MHz backscatter correlated with collagen deposition; however, the coefficient of correlation between myocardial collagen content and backscatter level was not available.

Using somewhat different techniques for analysis of backscatter amplitude (as opposed to the integrated backscatter), we found in six normal perfusion-fixed canine hearts that the average right ventricular backscatter amplitude was significantly higher than that from either the left ventricle or ventricular septum. We thought this finding might be related to collagen content, since previous studies (unrelated to ultrasound) have demonstrated that the collagen content of the right ventricle is approximately 30% higher than that of the left ventricle in normal human, porcine, bovine, and feline hearts. However, collagen content of the ventricles was not determined in our preliminary study, and the possibility of artifacts caused by the fixative or phase cancellation was not explored.

On the basis of these data, we postulated that the integrated ultrasonic backscatter from normal right ventricular myocardium should be higher than from the left ventricular myocardium because of the difference in collagen content. To test this postulate, we examined the relationship between regional integrated ultrasonic backscatter and collagen content in normal right and left ventricular myocardium in normal, freshly excised canine hearts and in normal, fixed human hearts.

Since only human hearts fixed in 10% formalin were available for backscatter measurements, a second purpose of our study was to evaluate the influence of fixation with 10% formalin on the measurements of integrated backscatter.

**Methods**

**Summary of protocols.** The ultrasonic backscatter from excised portions of normal canine and human left and right ventricles was studied with a 2.25 MHz center frequency transducer. Then the segment of myocardium corresponding to the ultrasound sample volume was assayed for hydroxyproline content. In addition, the ultrasonic backscatter from canine left ventricles was studied to compare backscatter in freshly excised hearts vs myocardium fixed with 10% formalin.

**Tissue preparation — dogs.** Ten adult mongrel dogs were anesthetized with pentobarbital. Asystole was induced by intravenous infusion of potassium chloride, and the hearts were rapidly excised. The hearts were examined to exclude gross pathologic changes and were then washed in 0.9% NaCl solution. The atria were excised and the left and right ventricular free walls were removed by sharp dissection along the ventricular septum. Both ventricular free walls and the ventricular septum were weighed for comparison with normal values. A 12 mm diameter region of interest for ultrasonic analysis was identified at the anterobasal left ventricle between the left anterior descending and circumflex coronary arteries. This region was free of large epicardial vessels and fat. Inkspots were placed on the epicardium through a template to define the region of interest (see below under Ultrasonic Measurements and figure 1). A similar procedure was performed at the corresponding level of the anterior right ventricle. Then 7 × 5 cm portions of the freshly excised canine right and left ventricles containing the region of interest were mounted side by side in a plastic frame and immersed in a 0.9% NaCl bath at 22° C with the epicardial surfaces facing an ultrasound transducer. Spontaneous fibrillations occurred in some ventricles upon immersion in the saline, and in these cases ultrasonic measurements were delayed a few minutes until such activity ceased. A suitable fragment of the remaining apical portion of the left ventricle was likewise mounted in a separate frame and maintained in saline before study in the fresh and fixed states.

After ultrasonic measurements, the paired right and left ventricular specimens were removed from the bath and the transmural regions of interest were excised. These were further dissected by sectioning off the epicardial layer (which gives rise to specular reflection upon ultrasound examination) and then by sectioning off the next 3 mm of mural myocardium. This resulted in a 400 to 500 mg wet weight fragment of myocardium that approximated the gated ultrasound sample volume. These fragments were quick-frozen on dry ice and stored at −10° C for subsequent hydroxyproline assay. All ultrasonic studies were completed within 55 min of excision of the hearts.

Next, the remaining mounted left ventricular specimen was also inked and positioned under the transducer, and ultrasonic

**FIGURE 1.** Ten inkspots were placed on the left ventricle between the left anterior descending (LAD) and left circumflex (LCx) coronary arteries and at the corresponding level of the right ventricle to define a region of interest. A 7 × 5 cm portion of each ventricle (interrupted line) containing the region of interest was excised and used for ultrasound measurements. Ao = aorta; PA = pulmonary artery; RC = right coronary artery; RV = right ventricle; LV = left ventricle.
measurements were performed. The frame containing the mounted specimen was then removed from the saline bath and immersed in a 10:1 volume of 10% acetate-buffered formalin for 2 weeks. During this period of fixation the color of the myocardium changed from brown to gray, and the myocardium became markedly stiffer. The shape did not change appreciably and the inkspots remained intact. After 2 weeks the frame containing the fixed ventricle was removed from formalin and repositioned in a water bath, and the ultrasonic measurements were repeated.

**Tissue preparation — humans.** Similar measurements of the right and left ventricular ultrasonic backscatter and collagen content were also performed in five male human hearts; these were obtained at postmortem examination (University of Iowa Hospitals, 1982 and 1983). Postmortem examination included determination of the heart’s weight, dissection of the coronary arteries, opening of the heart by cuts along both inflow and outflow tracts, and immersion fixation in 10% acetate-buffered formalin. Regions of the left and right ventricles were studied in a water bath by the same technique described above for the freshly excised canine ventricles, except that the fragment of myocardium corresponding to the gated ultrasound sample volume was stored in formalin, rather than frozen, before hydroxyproline assay. For inclusion as normal hearts in this study, the following criteria were met: no symptoms of heart disease (on chart review), cardiothoracic ratio of less than 0.5 on posteroanterior chest x-ray, no evidence of ischemic or hypertrophic heart disease on 12-lead electrocardiogram (available from three patients), no significant coronary arterial lesions, normal myocardium as determined from sections stained with hematoxylin and eosin, total heart weight less than 400 g, and ratio of left ventricular plus ventricular septal weight to right ventricular weight (epicardial fat removed) less than 3.4.15

**Ultrasonic measurements.** A 1.27 cm diameter, 2.25 MHz, 50% fractional bandwidth transducer with a 3 dB beam width of a 3.9 mm was used for all measurements (Panametrics Corp., Waltham, MA). The transducer was connected to a standard pulser-receiver, a stepless gate (both Panametrics), and an oscilloscope with a camera (Tektronix Corp., Beaverton, OR).

All data were taken in the far field of the transducer by positioning the transducer so that the initial deflection occurring at the epicardial specular reflection was at the transducer focal distance (6.1 cm from the transducer face). Measurements were obtained with a 4 μsec duration electronic gate beginning 2 to 4 μsec deep to the point of initial deflection of the epicardial reflection to exclude specular reflection (figure 2). A 4 μsec gate length (3.1 mm based on a velocity of sound in myocardial tissue) was used for all studies. A 2 μsec gate length was used for the 1982 canine myocardium.

**FIGURE 2.** Oscilloscopic displays of unprocessed radio frequency signals from the normal human left ventricle (top) and the normal right ventricle (bottom) are shown at a transducer center frequency of 2.25 MHz (5 μsec/division horizontal, 0.5 mV/division vertical). The average level of backscatter within the 4 μsec duration segment indicated by the brackets is higher in the right ventricle than in the left ventricle. Echoes from the left ventricular endocardial (EN) interface are often multiple and appear to correspond to trabeculation. EP = epicardial interface.
um of 1.57 mm/ìsec) was chosen for comparison of backscatter from the right and left ventricles in dogs and humans because of the relative thinness of the right ventricle compared with the left. Specular reflections from the epicardial interface were also excluded by this gate length. For comparisons of freshly excised left ventricles with those fixed with 10% formalin, an 8 ìsec gate (6.2 mm) was used. In all cases, the radio frequency signal within the gate was amplified, displayed on the oscilloscope at 1 ìsec/horizontal division, and recorded on Polaroid 667 film.

The photographs of the radio frequency signals were subsequently analyzed with a digitizing tablet (Summagraphics Corp., Fairfield, CT) and a PDP-11/34 computer (Digital Equipment Corp., Maynard, MA). The peaks and troughs in each radio frequency waveform within the gate were entered along with calibration data (figure 3). From these points, the entire waveform was reconstructed by linear interpolation between points. The reconstructed waveform was squared and integrated, yielding values of backscattered energy (E_{myocardium}). Reproducibility of the method was excellent, with a maximum variation of 2.5% among five determinations of backscatter from a photograph by the same observer.

For each study, a reference energy value (E_{steel}) was obtained from the surface reflection of a ½ inch thick flat polished slab of stainless steel that was substituted for the tissue at the same distance from the transducer. Integrated backscatter from myocardium was calculated in decibels down from the reflection of the water/steel interface by the formula

$$\text{Integrated backscatter} = 10 \log \left( \frac{E_{\text{myocardium}}}{E_{\text{steel}}} \right)$$

In each region of interest, multiple measurements were performed to minimize phase cancellation artifacts.\(^4\) It was assumed that the effects of phase cancellation were random and were reduced by averaging the results from 10 closely spaced, nonoverlapping sampling sites. Thus a region of interest was defined with a template to place 10 inkspots separated by 4 mm (the beam width) in three rows on the epicardial surface of the ventricle (figure 1).

Measurements were performed by placing the transducer over the region of interest and adjusting the transducer angle to obtain the maximal epicardial reflection (which occurred when the epicardial surface was approximately parallel to the transducer face). Each inkspot was centered in turn under the transducer by means of a mechanical guide and by movement of the tissue frame in the plane perpendicular to the ultrasound beam at the fixed distance (6.1 cm). Only slight changes in the magnitude of the angle-dependent specular reflection occurred with this procedure, which were attributed to curvature of the epicardial surface. The results of measurements at these 10 sites were averaged to obtain the integrated backscatter for each region of interest.

**Hydroxyproline assay.** Within 2 weeks of ultrasonic analysis, the frozen or fixed myocardium was taken to a constant dry weight. Although formalin (aqueous formaldehyde) is known to react with and precipitate many proteins, two previous studies have shown 10% formalin fixation does not affect the concentration of collagen in human myocardium.\(^{11,16}\) The dried samples were hydrolyzed in 6N hydrochloric acid at 110°C, and the hydrolysates were flash-evaporated. The neutralized residue was dissolved in a known volume of double-distilled water and passed through a 0.4 μm micropore filter (Gelman Instruments, Ann Arbor, MI).

Hydroxyproline analysis was based on a standard colorimetric technique.\(^{17}\) Hydroxyproline in an aliquot of the dissolved residue was oxidized with chloramine T, and the chloramine T was then destroyed upon addition of perchloric acid. Paradi-

![FIGURE 3. Display of unprocessed radio frequency signal from a normal human right ventricle (4 ìsec gate), indicating the method of signal analysis used in our study. After identification of the baseline (black dots and interrupted white line), the positions of the peak and trough voltage values (white dots) were digitized, along with calibration data. From these points (white dots), the waveform was reconstructed by linear interpolation. The values were then squared and integrated to yield integrated ultrasonic backscatter.](http://circ.ahajournals.org/)-methylaminobenzoic acid reagent was added, and a chromagen was produced upon heating for 20 min at 60°C. Duplicate myocardial aliquots and five dilutions of a standard solution were tested for absorbance at 557 nm. Results are expressed as micrograms of hydroxyproline per milligram dry weight of the tissue. Our results can be compared with other values in the literature by noting that 7.46 times hydroxyproline concentration equals collagen concentration and about one-fourth wet weight equals the dry weight.\(^{12,18}\) Therefore the conversion can be accomplished with the formula

$$\frac{\mu g \text{ collagen}}{mg \text{ wet weight}} = \frac{\mu g \text{ hydroxyproline}}{mg \text{ dry weight}} \times \frac{7.46}{4}$$

In addition, it should be noted that approximately 12% of collagen is lost upon removal of the epicardium and endocardium.\(^{12}\)

**Statistical analysis.** Student’s paired t-test was used to test the significance of differences found between (1) the right and left ventricular integrated backscatter in canine and human hearts, (2) the right and left ventricular hydroxyproline content in canine and human hearts, and (3) the integrated backscatter in freshly excised canine left ventricles and those fixed with 10% formalin. Linear regression analysis (least squares method) was used to obtain the coefficients of correlation for the integrated backscatter vs the hydroxyproline content calculated separately for canine and human hearts.
Results

Ultrasonic measurements. In canine hearts, the integrated backscatter (4 μsec gate) from the region of interest in the left ventricle was $-66.9 \pm 1.0$ (SEM) dB and was significantly lower than the right ventricular average of $-60.4 \pm 1.6$ dB ($p < .001$, figure 4). Findings in human hearts were similar, with an average backscatter from the left ventricle of $-65.1 \pm 0.9$ dB, significantly lower than the right ventricular average of $-60.2 \pm 1.2$ ($p < .005$, figure 4). The integrated backscatter was of a similar magnitude in canine and human hearts. Since the standard error of the means was much less than the observed differences in the means, 10 measurements per region were probably sufficient to minimize the effects of phase cancellation.

In canine hearts, the integrated backscatter (8 μsec gate) from the freshly excised left ventricle was $-64.8 \pm 0.7$ (SEM) dB. This latter value of $-64.8$ dB for freshly excised left ventricle (8 μsec gate) is somewhat greater than the value previously given ($-66.9$ dB with a 4 μsec gate) because of the difference in gate length. When corrected for gate length, no substantial difference for backscatter was found. A modest decline of backscatter to $-65.6 \pm 0.7$ dB occurred after 2 weeks of 10% formalin fixation ($p = \text{NS}$, figure 5).

Hydroxyproline content. In canine hearts, the average hydroxyproline content from the region of interest in the left ventricle was $3.58 \pm 0.13$ (SEM) μg hydroxy-

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**FIGURE 4.** Both backscatter and hydroxyproline determinations were higher in the right ventricle than in the left ventricle of 10 canine hearts (A) and five human hearts (B).
proline/mg dry weight and was significantly lower than the right ventricular average of 4.40 ± 0.26 μg/mg (p < .005, figure 4). Human hearts demonstrated a similar trend, with an average hydroxyproline content in the left ventricle of 5.19 ± 0.27 μg/mg, significantly lower than the right ventricular average of 7.45 ± 0.61 μg/mg (p < .05, figure 4).

Unlike the integrated backscatter, average hydroxyproline content was considerably higher in human than in canine myocardium. This finding is consistent with the values obtained for canine left ventricle by Mimbs et al.4 (collagen = 0.8% wet weight) and for formalin-fixed human left ventricle by Caspari et al.11 (collagen = 40.7 mg/g dry weight).

**Correlation of backscatter and hydroxyproline content.** There were no significant coefficients of correlation between backscatter and hydroxyproline content. The coefficient of correlation for hydroxyproline content vs the integrated backscatter in all regions of human hearts was r = .32 (p = NS, figure 6). Similar results were found in canine hearts (r = .28, p = NS, figure 6).

**Discussion**

The main findings of this study were: (1) in normal canine and human ventricular myocardium, both ultrasonic backscatter and hydroxyproline (collagen) content were higher in the right ventricle than in the left; (2) there was no significant correlation between the integrated backscatter and the hydroxyproline content; and (3) formalin fixation did not appear to substantially alter the ultrasonic backscatter compared with freshly excised myocardium.

It should be noted that our values of integrated backscatter are somewhat lower than those reported previously by other investigators. This difference is due in part to our use of a lower center frequency transducer (2.25 MHz), compared with the 5 MHz center frequency transducer used by Mimbs et al.4 In addition, we used a transducer with a narrower fractional bandwidth (50%) compared with the 75% to 100% bandwidth transducers used by others.5 Since ultrasonic backscatter is directly related to frequency, a lower value for integrated backscatter is to be expected with our method.

We found an average of 23% more collagen per unit weight in the right ventricles than in the left ventricles of canine hearts, and this was associated with a 6.5 dB increase in backscatter. This difference in collagen content is comparable to the 18% increase of collagen in the “myopathic” left ventricles of the doxorubicintreated rabbits studied by Mimbs et al.9 This increase in collagen was associated with a significant increase of 4 dB in the 5 MHz backscatter.9 This apparent relationship of increased collagen to higher levels of

![FIGURE 5](Image)

**FIGURE 5.** In 10 freshly excised canine left ventricles, there was no significant change in the regional integrated backscatter after 2 weeks of fixation in 10% formalin.

![FIGURE 6](Image)

**FIGURE 6.** There was no significant correlation between integrated backscatter and hydroxyproline concentrations in 10 canine hearts (A) or five human hearts (B). Filled circles, right ventricle; open circles, left ventricle.
backscatter in our data and in the other studies cited is consistent with the hypothesis that collagen, at least in part, determines ultrasonic backscatter. The association of higher values for collagen and backscatter without a strong correlation between these two variables suggests that structural collagen is not the only factor influencing backscatter from normal myocardium; other myocardial constituents may also interact with ultrasound. Variations of regional ultrasonic indexes might occur independently of collagen content because of the anisotropy of ultrasound propagation in muscle. This may have contributed to the lack of correlation between backscatter and collagen content over the narrow range of collagen content found in the normal hearts we studied.

There is little doubt that collagen deposition is an important influence on the integrated backscatter from abnormal myocardium. The specificity of increased backscatter for the diagnosis of myocardial fibrosis, however, is uncertain. For example, large differences in backscatter were found between normal right and left ventricles in our study. Furthermore, an increase of 6.3 dB in the integrated backscatter was observed 6 hr after acute infarction in canine hearts, before the stage of fibroblastic repair.

From a clinical standpoint, interpretation of backscatter data obtained at a single point in time without knowledge of previous values for that patient might be difficult. The level of backscatter would be expected to vary depending on the amount of attenuation, phase cancellation, and other effects introduced by tissues interposed between the transducer and the region of interest. Evaluation of the ratio of right ventricular to left ventricular backscatter might provide a useful approach when changes in right and left ventricular collagen content are independent. The ratio of right to left ventricular backscatter in each of the normal hearts we studied was greater than 1. Values less than 1 (2 SDs below the mean) might be indicative of left-sided fibrosis, irrespective of changes introduced by the intervening chest wall.

The effects of fixation on the ultrasonic properties of tissue are of interest to investigators, since fixed tissues are easier to cut, easily transported, may be studied without ongoing postmortem changes, and can be stored for long periods. Although the velocity of sound in myocardium fixed with 10% formalin is faster than that in freshly excised myocardium, data concerning the influence of fixation on myocardial ultrasonic backscatter were not previously available. After 4% formalin fixation, a modest decline in the level of backscatter at 2.25 MHz from freshly excised human and bovine liver was found by Bamber et al. We found that myocardial integrated backscatter is not significantly changed by formalin fixation, suggesting the myocardial elements that cause backscatter of ultrasound are likewise not altered by formalin fixation.

We conclude that ultrasonic backscatter varies within normal myocardium, with the backscatter from the right ventricle higher than that from the left ventricle. In canine and human hearts, this is associated with a difference in collagen content between the ventricles. Finally, 10% formalin is a suitable fixative for ultrasonic studies of the myocardium in vitro.

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