Ultrasonic Detection of the Anisotropy of Protein Cross-Linking in Myocardium

Steven L. Baldwin, Min Yang, Karen R. Marutyan, Kirk D. Wallace, Mark R. Holland, and James G. Miller
Department of Physics, Washington University in St. Louis, MO 63130

Abstract— **Diastolic dysfunction may arise, in part, because of an increase in myocardial stiffness from cross-linking of extracellular matrix proteins such as collagen. The goal of the current study was to measure changes in myocardial attenuation resulting from increased protein cross-linking as a function of the angle of insonification relative to that of the predominant myofiber orientation. Increased cross-linking inferred from chemical fixation in formalin resulted in a maximum increase in attenuation at perpendicular insonification. This study indicates that effects of fixation, and therefore presumably protein cross-linking, in myocardium can be anisotropic, suggesting that the response of the extracellular collagenous matrix to changes in cross-linking is directionally dependent. The anisotropy of ultrasonic attenuation may thus provide an approach for the noninvasive monitoring of the extent and progression of myocardial disease associated with changes in protein cross-linking.**

I. Introduction

Increased myocardial stiffness that may result in specific cardiac pathologies such as diastolic dysfunction has been attributed to an increase in cross-linking of extracellular matrix proteins such as collagen [1-4]. These changes are a result of glycation of proteins and the formation of advanced glycation end-products (AGEs) that cause cross-linking of collagen molecules to each other and a corresponding increase in tensile stiffness [1]. Studies have shown that AGE-related cross-links increase as a normal part of the aging process [5, 6] and have suggested that they are accelerated by the existence of elevated concentrations of glucose in patients with diabetes [1, 3]. Recent work has focused on therapeutic approaches which break collagen cross-links in an effort to ameliorate the adverse cardiovascular changes associated with aging, diabetes, and hypertension [3, 7, 8]. With the growing body of knowledge regarding the role of protein cross-linking in myocardial disease and the development of new approaches to cardiovascular therapy, it becomes increasingly valuable to develop noninvasive approaches for monitoring changes in protein cross-linking both *in vitro* and *in vivo*.

Previous studies have suggested that ultrasonic attenuation may be influenced by the amount of collagen in soft tissues [9, 10]. Work by Hall *et al.* examined the use of high frequency ultrasound (30 to 50 MHz) in acoustic microscopy to detect changes in protein cross-linking in rat hearts induced by chemical fixatives (formalin and glutaraldehyde) [11]. It has been suggested that increased cross-linking of collagen within the myocardium caused by chemical fixatives such as formalin could mimic some of the more complex changes in the cardiac fibrous matrix that occur with aging, hypertension, or diabetes [11]. The study by Hall *et al.* showed a progressive monotonic increase in the ultrasonic attenuation as a function of the temporal extent of protein cross-linking for insonification perpendicular to the predominant direction of the myofibers [11]. Healthy myocardium has been shown to exhibit anisotropy with respect to ultrasonic attenuation [12, 13], velocity [14], and backscatter [15]. For this reason, the primary objective of this study was to determine whether changes in ultrasonic attenuation induced by the effects of increased protein cross-linking might also depend on the angle of insonification.

II. Methods

A. Specimen Preparation

A total of 36 tissue specimens from 12 freshly excised lamb hearts were investigated. The hearts were obtained within 30 minutes of slaughter from a local commercial slaughterhouse and immersed in 0.9% saline solution at room temperature prior to specimen coring. From each ovine heart, three cylindrical plugs were cored, two from the left ventricular free wall and one from the septum, using a 14.5 mm inner-diameter coring tool. Specimens were cored from endocardium to epicardium such that the axis of symmetry of the cylindrical specimens was oriented orthogonal to the epicardial surface of the heart. The two left ventricular free wall specimens were cored in approximately the same plane perpendicular to the apex-to-base axis of the heart and superior to the anterior and posterior papillary muscles. The septal specimens were cored approximately half way between the plane of the left ventricular specimens and the apex. This standardized method of coring provided for a regional comparison of the anisotropy of the myocardial specimens. Epicardial surfaces were trimmed of excessive fat, if deemed necessary, prior to being glued to a plastic mount of equivalent diameter for data acquisition.

B. Data Acquisition and Analysis

Each myocardial specimen was cored from an intact left ventricle at room temperature that had been stored in the saline solution to reduce the potential for osmotic effects. Immediately after coring, the specimen was mounted and then immersed in a water bath (also at room temperature)

approximately 5 to 10 minutes prior to measurements. The total time from death of the animal to completion of all measurements on that particular heart was approximately three to five hours. Temperature changes during any one data acquisition were no more than 0.1˚C. For 27 out of 36 freshly excised ovine specimens measured, the temperature was maintained between 19.1 and 20.1˚C. For the remaining 9 ovine specimens, temperature extremes were 17.6˚ to 19.1˚ C. Mean results excluding these specimens were not significantly different. For all 36 formalin-fixed ovine specimens measured, the temperature was maintained between 19.0˚ and 20.1˚ C.

Immediately following measurements of freshly excised myocardium, specimens were immersed in formalin to induce protein cross-linking. After sufficient time had elapsed to ensure that the process of cross-linking had reached completion (at least one month), measurements were repeated on the identical specimens. Fig. 1 shows the equipment setup for measurements. The through-transmission radiofrequency data were acquired using a matched pair of 5 MHz focused piezoelectric transducers (Panametrics V309, 1/2 inch diameter; Panametrics, Waltham, MA) separated by twice the nominal focal length of 2 inches. The tissue specimens were positioned at the focus of both transducers and the ultrasonic beam was centered on the midmyocardial region of each specimen. An electric pulse was generated with a Panametrics 5800 Pulser/Receiver operating in pitch/catch mode and sent to the transmit transducer. The through-transmitted signal was detected by the receive transducer, amplified by the Panametrics 5800, and digitized with a Tektronix 2430A (Tektronix, Beaverton, OR) oscilloscope.

For data acquisition, the plastic mount of each myocardial specimen was friction fit into a cylindrical plastic coupling that was, in turn, attached to the metal shaft of the rotational axis. A Unidex 12 motion controller (Aerotech, Pittsburgh, PA) was utilized to permit the measurement assembly to have x, y, and z translational movements as well as rotational freedom. A computer (Apple Computer, Cupertino, CA) served as a data acquisition system controller and off-line storage device for digitized data. Each myocardial specimen was rotated in 5˚ increments under computer control. Substitution through-transmission data were obtained by averaging 32 radiofrequency traces at each incremented angle over a complete rotation. In addition, immediately after through-transmission data acquisition, pulse-echo data were acquired for specimen thickness measurements at each incremented angle. Corresponding water path reference data were obtained to within ± 0.1˚C of the corresponding specimen data.

Ultrasonic measurements of thickness were performed at each angle of rotation for each myocardial specimen. This approach has been described previously [14].

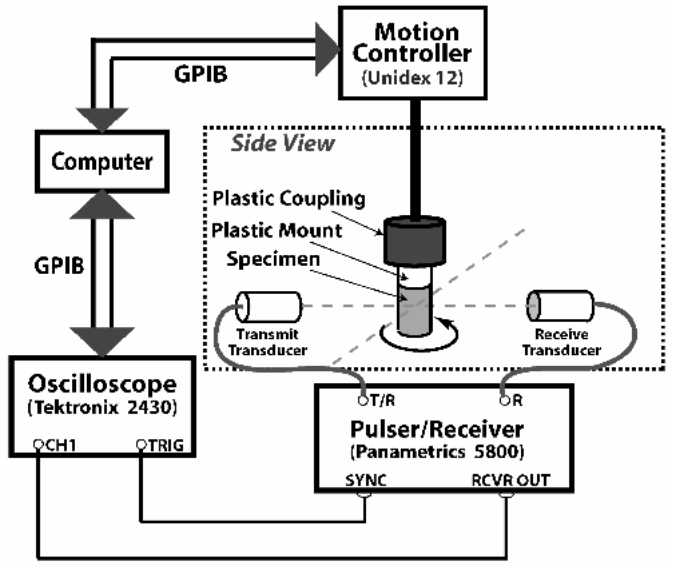

Fig. 1. Experimental setup for performing measurements of the anisotropic properties of myocardial attenuation.

III. Results

For both freshly excised and formalin-fixed myocardium, the attenuation varied systematically as a function of angle of insonification, with a minimum perpendicular and a maximum parallel to the direction of the myofibers. In both cases, the attenuation coefficient was found to increase as a function of frequency in an approximately linear manner. Fig. 2 shows the mean attenuation coefficient at 5 MHz expressed in nepers/cm. In contrast, *changes* in attenuation as a consequence of chemically-induced protein cross-linking show a different directional (angular) behavior. Formalin-fixation resulted in a maximum increase in attenuation at perpendicular insonification.

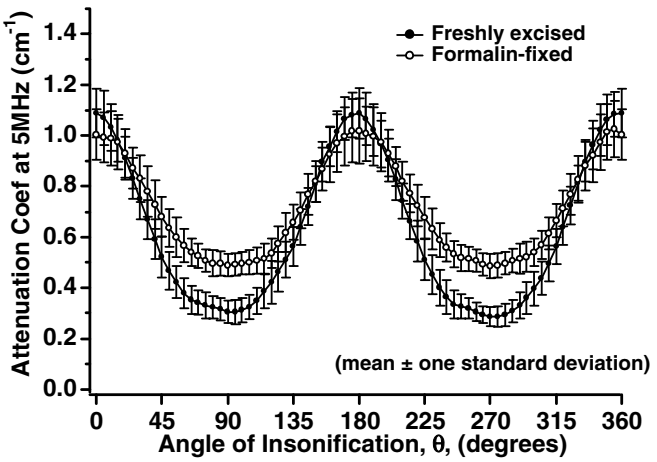

Fig. 2. Mean results as a function of angle of insonification for freshly-excised and formalin-fixed myocardium for attenuation coefficient at 5 MHz (cm^{-1}).

For angles near perpendicular to the predominant direction of the myofibers, the average slope of attenuation increased from 0.52 ± 0.07 dB/(cm•MHz) (mean ± one standard deviation) for freshly excised to 0.85 ± 0.08 dB/(cm•MHz) for formalin-fixed myocardium. In contrast, as angles approached parallel insonification, less change resulting from cross-linking was apparent. At parallel insonification, measurements resulted in averages of 1.88 ± 0.17 for freshly excised and 1.75 ± 0.19 dB/(cm•MHz) for formalin-fixed myocardium.

The data presented in Fig. 2 indicate that the attenuation depends on the angle between the direction of insonification and the predominant direction of the myofibers. As the myocardial specimens are rotated by 360˚, the angle between the direction of insonification and the predominant direction of the myofibers varies back and forth between 0˚ and 90˚ a total of four times. In Fig. 3, data over the full 360˚ acquisition have been averaged accordingly in order to show the mean *slope* of attenuation (± one standard deviation) for freshly excised and formalin-fixed myocardium. The percentage increase in mean slope of attenuation resulting from increased protein cross-linking ranges from near zero at angles close to parallel to approximately 63% for angles near perpendicular insonification. A comparison of results from different regions of the left ventricle and septum shows no significant regional differences in slope of attenuation for all angles of insonification for either freshly excised or formalin-fixed myocardium.

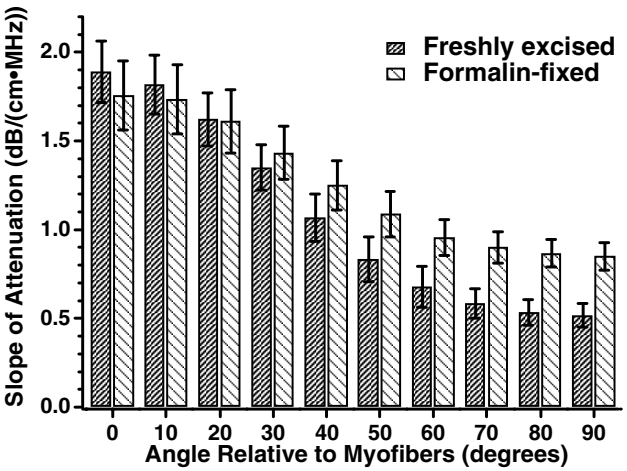

Fig. 3. Comparison of mean slope of attenuation for freshly excised and formalin-fixed myocardium averaged as a function of angle relative to the myofiber direction (mean ± one standard deviation).

IV. Discussion

Collagen cross-linking occurs on the molecular level and involves intermolecular cross-links among the collagen molecules that make up collagen fibrils [5]. The chemical fixative employed in this study as a model for induced cross-linking, formaldehyde, is an efficient and nonspecific producer of protein-protein cross-links among proteins that are in close physical proximity (2 Å) [16]. Furthermore, previous studies have indicated that formalin-fixation does not influence collagen content, type, or significantly alter the organization (micro-architecture) of the collagen fibers that comprise the extracellular matrix [17-19]. Thus, changes in myocardial attenuation observed in the current study resulting from formalin-fixation might be expected to primarily affect the material properties of the existing extracellular collagen matrix.

Given the complex structure of myocardium and the action of formalin in protein cross-linking, the current study may offer insight into the nature (mechanisms) of ultrasonic attenuation in the heart. Previous studies have investigated the dependence of myocardial attenuation on the variable of angle of insonification relative to the myofiber direction [12]. Provided that chemically-induced protein cross-linking primarily affects myocardial attenuation through changes in the material properties of the existing extracellular collagen matrix, the current study may permit the direct investigation of this new variable at a complete range of angles of insonification. It is interesting to observe that measured changes in myocardial attenuation resulting from altering the degree of protein cross-linking are not proportional to changes in attenuation as a function of angle of insonification. As seen in Fig. 2 and Fig. 3, *changes* in attenuation have maxima at angles where the myocardial attenuation exhibits minima and appear to have minima where the myocardial attenuation exhibits maxima. These results may suggest that measured attenuation in myocardium depends upon differences in the response of the anisotropic collagen fiber architecture and relative collagen type to the local degree of cross-linking.

This study has shown that the effects of chemically-induced protein cross-linking on myocardial attenuation result in a monotonic increase as the angle of insonification is incremented from parallel to perpendicular to the predominant direction of the myofibers. A knowledge of how anisotropic ultrasonic properties change in response to disease may suggest novel approaches for diagnosis, for example, of pathologies associated with protein cross-linking in myocardium. Measurements over a full range of angles can be obtained in ultrasonic studies carried out *in vitro*. These investigations can provide the basis for proper interpretation of studies *in vivo*, in which certain factors (such as available ultrasonic windows through the chest wall) limit the positions and orientations from which ultrasonic data can be obtained.

The measured increase in attenuation for perpendicular insonification relative to the predominant myofiber direction is consistent with results from earlier work. In the previous high frequency study (30-50 MHz) by Hall *et al.* that examined the time evolution of protein cross-linking in rat myocardium at perpendicular insonification, the slope of attenuation in formalin-fixed tissue showed an increase of 75% over that measured in freshly excised tissue [11]. In comparison, results of the current study (3-7 MHz) in lamb hearts showed a 63%

increase in slope of attenuation at perpendicular insonification.

V. CONCLUSIONS

This study has shown that effects of increased protein cross-linking on myocardial attenuation depend on the direction of insonification relative to the predominant direction of the myofibers. This may have significant implications for the use of ultrasound in detecting changes in myocardial protein cross-linking related to disease. Results suggest that attention to the anisotropy of attenuation may be essential for the use of ultrasound in detecting physiological or pathological changes in collagen cross-linking in myocardium. In the model for cross-linking employed in this study, formalin-fixation results in clear changes in attenuation for insonification near perpendicular to the myofiber direction. However, little or no change would be observed when insonifying parallel to the myofiber direction which suggests that at certain angles, changes associated with disease or drug therapy may incorrectly go undetected. The changes in ultrasonic attenuation with angle associated with protein cross-linking in myocardium may offer potentially useful diagnostic information about changes in mechanical properties as a function of angle of insonification.

ACKNOWLEDGMENT

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