High Frequency Acoustic Parameters of Human and Bovine Articular Cartilage Following Experimentally-Induced Matrix Degradation


1Department of Medical Biophysics, University of Toronto
Sunnybrook and Women's College Health Sciences Centre
2075 Bayview Avenue, Toronto, ON, Canada M4E 1Y3
stuart.foster@swchsc.on.ca

2Department of Surgery
St. Michael's Hospital
Toronto, ON, Canada

3Department of Pathology
Mt. Sinai Hospital
Toronto, ON, Canada

4Department of Chemical Engineering and Institute of Biomedical Engineering
Ecole Polytechnique de Montréal
Montréal, QC, Canada

Matrix degradation and proteoglycan loss in articular cartilage are features of early osteoarthritis. To determine the effect of matrix degradation and proteoglycan loss on ultrasound propagation in cartilage, we used papain and interleukin-1a to degrade the matrix proteoglycans of human and bovine cartilage samples, respectively. There is also minor collagen alteration as associated with these chemical degradation methods. We compared the speed of sound and frequency-dependent attenuation (20-40 MHz) of control and experimental paired samples. We found that a loss of matrix proteoglycans and collagen disruption resulted in a 20-30% increase in the frequency-dependent attenuation and a 2% decrease in the speed of sound in both human and bovine cartilage. We conclude that the frequency-dependent attenuation and speed of sound in articular cartilage are sensitive to experimental modifications of the matrix proteoglycans and collagen. These findings suggest that ultrasound can potentially be used to detect morphologic changes in articular cartilage associated with the progression of osteoarthritis.

KEY WORDS: High frequency; osteoarthritis; proteoglycan; tissue characterization; ultrasound; ultrasound biomicroscopy.

INTRODUCTION

Osteoarthritis is a common degenerative disease, which affects the articular cartilage of weight-bearing joints. The progression of osteoarthritis involves a progressive and slow degradation of the articular cartilage, which can lead to complete loss of tissue and osteophyte formation. The cartilage degradation is characterized by a decrease in tissue thickness, remodeling of the collagen matrix and proteoglycan content, as well as a change in cellular organization. Osteoarthritis is characterized by a loss of matrix proteoglycans and edema of the cartilage.

Previous investigations using high-frequency ultrasound tissue characterization have demonstrated that chemically-induced removal of matrix proteoglycans in bovine cartilage leads to changes in the frequency-dependent attenuation and the speed of sound. It has
also been shown that experimentally-induced matrix degradation can alter the ultrasound backscattered signal from bovine and rat cartilage. Recently, Töyräs et al. have shown that exposure of articular cartilage to matrix-degrading enzymes, chondroitinase ABC and collagenase, results in changes to the echogenicity as well as the speed of sound. Töyräs et al. also observed a decrease in the Young's modulus following degradation, indicating a relationship between the matrix constituents and mechanical properties of cartilage. Previous investigations into the effects of matrix degradation on frequency-dependent attenuation and speed of sound performed on human articular cartilage had difficulty in showing significant changes in speed of sound or attenuation. This is most likely attributed to the measurement techniques and frequency used. At present, there is a lack of data on the frequency-dependent attenuation in the 20 to 40 MHz range in normal human and bovine articular cartilage.

The purpose of this study was to determine the effect of experimentally-induced matrix degradation and collagen alteration on the speed of sound and frequency-dependent attenuation in human and bovine articular cartilage in the 20 to 40 MHz range. Osteoarthritis-like changes were induced in human and bovine articular cartilage samples using two independent models. Human cartilage was digested in papain, an enzyme which degrades the matrix proteoglycans of articular cartilage while imparting minor damage to the collagen network at low concentrations. Bovine cartilage explants were incubated in interleukin-1α, a cytokine that promotes the release of collagen and proteoglycan specific proteinases by the chondrocytes. We performed ultrasound tissue characterization on these two models of experimental matrix degradation and compared the changes in the acoustic parameters between control and matrix degraded cartilage specimens. Results demonstrating the relationship between the acoustic parameters and matrix proteoglycan and collagen degradation are presented.

MATERIALS AND METHODS

In vitro digestion of human articular cartilage with papain

Samples of "normal" cartilage used in this study were obtained at autopsy from the knees of two healthy, 29 and 34-year-old patients. This cartilage was obtained from the bone bank at Mt. Sinai Hospital (Toronto, Canada). Articular cartilage was stored at -30°C prior to sectioning. It was shown in various tissues that no significant changes in the speed of sound, attenuation or backscatter were found in measurements made in a fresh sample compared with the same sample after it had been frozen and thawed. Femoral condyles were sectioned into cartilage pieces 5 mm in lateral direction and care was taken were from the subchondral bone with a scalpel. These samples were referred to as "young normal cartilage." Seventeen young, normal cartilage specimens were obtained for control vs. digested experiments. Seven of these young normal specimens served as healthy normal control specimens, while ten samples were digested in various concentrations of papain to obtain a dose response of the acoustic parameters to papain digestion.

A second group of human articular cartilage specimens were obtained from the femoral condyles of 24 patients, 18 female and 6 male, undergoing total knee arthroplasty for osteoarthritis. These samples were selected from regions of the condyles that were visually judged to be the least affected by osteoarthritis. These samples were referred to as "control" cartilage.

The mean age of the patients was 71 ± 7 years of age. Femoral condyles were cut into 24 pairs of samples of 5 mm width, adjacent to one another, to serve as digested and control.
FIG. 1 (a) Photomicrograph of a control section of decalcified human articular cartilage stained with Safranin O (darker range of grayscale). (b) Significant reduction in proteoglycan staining is observed in a sample of human cartilage treated in 3% papain for 2 hrs. Bar = 1 mm.

Specimens. Adjacent samples were chosen in order to provide a good control for the experiment.

For each human sample pair, the experimental specimen was incubated in a solution of 0.3% papain (Sigma P-3125), while the control was incubated in a 0.3% solution of 0.05 M sodium acetate (Sigma S-7899) and 0.01% thymol (Sigma T-0501). Both specimens were incubated for 12 hours at 37 °C. Following digestion, the specimens were removed from solution and washed with distilled water. A portion of each specimen underwent proteoglycan specific Safranin O staining, to provide our qualitative measure of proteoglycan loss, as described by Lillie. The remainder of the specimens were stored at 5 °C in humid containers for up to 24 hours prior to ultrasonic tissue characterization.

Ultrasonic tissue characterization was performed on human articular cartilage samples for papain concentrations in the range of 0.005% to 0.5%. We observed consistent changes in the normalized attenuation (increase) and speed of sound (decrease) for papain concentrations greater than 0.1%. Hence, for our model of inducing osteoarthritis-like changes in cartilage with papain, we chose a digestion concentration of 0.3% (12 hrs.) for further experiments with this experimental model of osteoarthritis. Safranin O histology confirmed the removal of proteoglycans from human cartilage specimens digested with 0.3% papain. Safranin O stained photomicrographs of control and papain-digested human cartilage specimens are presented in figures 1a and 1b. Figure 1a shows the control sample with strong Safranin O staining (Orange) indicating the normal level of proteoglycan concentration in the cartilage matrix. Figure 1b shows a loss of Safranin O intensity, following papain-digestion, associated with a loss of cartilage matrix proteoglycans.
Bovine cartilage explants cultured with interleukin-1a

Normal bovine cartilage was obtained fresh. Two stifle joints from young steers, with intact synovial capsules, were selected for use and stored at 0 °C during transport to prepare the cartilage and chondrocytes in their natural environment. A total of 15 pairs of 3 mm diameter and 1.5 mm thick cartilage explants were isolated and prepared for culture according to the methods described by Dumont et al. Tissue culture experiments were performed in Montréal, Quebec. Each pair had one sample cultured in interleukin-1α, while the other served as a control. Experimental cartilage explants were cultured in serum-free media, along with 5 ng/ml of recombinant human interleukin-1α, while control explants were cultured in serum-free media alone. All explants were incubated at 37 °C and 5% CO₂ for 11 days. Following incubation, three sample pairs were immediately subjected to mechanical testing in order to determine equilibration and dynamic stiffness, as described by Soulhat et al. These three sample pairs were also assayed with Farndale's dimethylmethylene blue method to determine the extent of proteoglycan removal in the cartilage explants. The remaining 12 sample pairs were stored in humid conditions at -30 °C during transport from Montreal to Toronto. Upon arrival, samples were stored at 5 °C for 12 hours awaiting ultrasonic tissue characterization.

To use the culture of bovine cartilage in interleukin-1α as an independent model of matrix degradation and proteoglycan loss, we needed to validate the effects of the interleukin-1α on matrix cartilage proteoglycans. The glycosaminoglycan (GAG) content present in the control and dexamethasone samples was measured using Farndale's dimethylmethylene blue method. In three control samples, the mean GAG weight was 680 ± 260 mg whereas in three interleukin-1α cultured samples, the average GAG weight was 155 ± 55 mg. Thus, there was a mean loss of 80% of the proteoglycan content following digestion, confirming the effectiveness of our interleukin-1α model of matrix degradation.

Ultrasonic tissue characterization

The methods used for calculating the ultrasonic frequency-dependent attenuation and speed of sound were based on those previously described by D’Astous and Foster. The setup consists of a radio frequency (RF) pulser (Avtech Inc., Ottawa, Canada) and a 30 MHz piezoelectric transducer manufactured in our laboratories, as well as electronics that have been previously described. Prior to ultrasonic tissue characterization, the cartilage specimens were cut, in order to detach the articular cartilage from the underlying bone. These small cartilage plugs were placed in a sample holder, consisting of a quartz reflector plate and a resinite cover film (Borden Packaging, Canada) to hold the sample in place, as shown in figure 2. The entire tissue mounting stage was kept in 1% degassed phosphate buffered saline (PBS) at 37 °C throughout the experiment.

Data acquisition consisted of collecting a raster pattern (350 mm x 350 mm) of 64 RF lines, with 50 mm separation between individual acquisition points. The fine motion of the transducer was controlled by a PC computer and a Burleigh 7000 micropositioning system (Burleigh Inc., Rochester, NY). The RF lines were collected by a HP54201D digital oscilloscope (Hewlett-Packard) and stored on hard disk for further analysis. Data analysis is performed using Matlab software using the algorithms of D’Astous and Foster to calculate frequency-dependent attenuation at a frequency in the 20 to 40 MHz frequency range as well as the speed of sound in cartilage specimens. Speed of sound was calculated using pulse-echo time of flight, while frequency-dependent attenuation coefficients were measured using the
insertion-loss technique. Once calculated, the frequency-dependent attenuation coefficients were fitted to

$$\alpha = \alpha_0 f^\gamma \text{dB mm}^{-1} \text{MHz}^{-1} \quad (1)$$

where \(\gamma\) is the frequency-dependent factor in the attenuation coefficient, and \(\alpha_0\) is a constant.

Data analysis

Data are summarized as mean ± standard error in the mean, unless otherwise indicated. To analyze the changes in the results from the control and experimental cartilage samples, we performed a paired Student's t-test on measurements of the frequency-dependent attenuation and the speed of sound. To compare the changes in the frequency-dependent attenuation between the young normal cartilage and the control cartilage specimens, we performed a Student's t-test because the two populations were not related. We considered differences significant at \(p < 0.05\).

RESULTS

Acoustic parameters of human cartilage

Results for speed of sound and attenuation measurements in human cartilage are given in figures 3 and 4 respectively. The measured value of the speed of sound for the seven young normal samples was 1666 ± 16 m/s and was very close to the measured speed of sound in the controls (1664 ± 7 m/s) as shown in figure 3. These values are in good agreement with the results from the literature characterizing the speed of sound at 1665 m/s in human articular cartilage. The mean speed of sound was 2% lower in the 24 proteoglycan-depleted carti-
FIG. 3 Effect of matrix proteoglycan degradation on speed of sound (mean ± SEM) for young normal (n=7), control (n=24) and papain digested (n=24) human cartilage samples.

FIG. 4 Frequency-dependent attenuation (mean ± SEM) for 24 control and papain-digested human cartilage samples. Frequency-dependent attenuation data for seven young normal human cartilage samples are also presented.

The observed mean decrease of the speed of sound was statistically significant, p = 0.03.

A summary of the frequency-dependent attenuation data for the 24 paired control and papain-digested human cartilage samples along with the seven young normal cartilage samples is presented in figure 4. The mean attenuation coefficient of the young cartilage, measured at 30 MHz, was 6.2 ± 0.4 dB/mm while that of the control group was 7.1 ± 0.4 dB/mm. The corresponding value in the 24 papain-digested cartilage samples was 20% higher than in
FIG. 5 Effect of matrix proteoglycan degradation on the speed of sound (mean ± SEM) in 12 control and interleukin-1 cultured bovine cartilage samples.

FIG. 6 Frequency-dependent attenuation (mean ± SEM) for 12 control and interleukin-1 cultured bovine cartilage samples. The corresponding control cartilage samples (8.5 ± 0.5 vs. 7.1 ± 0.4 dB/mm). The increase of the mean frequency dependent attenuation was statistically significant, p = 0.02. The data for all populations were fitted to Eq. (1) and these lines of best fit are also displayed in figure 4.

Tissue characterization of bovine cartilage explants cultured in interleukin-1a

The mean speed of sound was 2% lower in the 12 interleukin-1 cultured bovine cartilage samples than in the 12 corresponding control bovine cartilage samples (1631 ± 17 vs. 1666 ± 8 m/s). The observed decrease of the speed of sound was statistically significant, p =...
A summary of the speed of sound data for the 12 paired control and proteoglycan-depleted bovine cartilage samples is presented in figure 5. The mean attenuation coefficient, measured at 30 MHz, was 30% higher in the 12 interleukin-1 cultured bovine cartilage samples than in the 12 corresponding control cartilage samples (9.1 ± 1.0 vs. 6.8 ± 1.2 dB/mm). The observed mean increase of the frequency-dependent attenuation was statistically significant, p = 0.04. A summary of the frequency-dependent attenuation data for the 12 paired control and proteoglycan-depleted bovine cartilage samples is presented in figure 6.

**DISCUSSION**

Our experiments in human and bovine articular cartilage show that matrix degradation and proteoglycan loss results in a decrease in the speed of sound and an increase in the frequency-dependent attenuation. We used two independent experimental models of osteoarthritis: digestion of human cartilage with papain and culture of bovine cartilage with interleukin-1a. Both models function by primarily degrading the proteoglycans, although the mechanism of degradation for each method is different. Previous researchers have reported the speed of sound in normal human cartilage to be 1665 m/s. Our results of 1666 ± 16 m/s conform to these previously published values. The 2% (p < 0.05) decrease observed in our speed of sound data for human and bovine cartilage, following matrix degradation and proteoglycan loss, is consistent with the observations of Myers. Myers also reported a 4% mean decrease in the speed of sound when comparing osteoarthritic and normal human cartilage specimens; however, they did not attempt to establish a significant correlation between the speed of sound in normal and osteoarthritic cartilage specimens. Conversely, the data available for bovine cartilage, measured at 100 MHz, indicates a slight decrease in the speed of sound after depleting bovine cartilage of chondroitin sulfate, a glycosaminoglycan or collagen. The observed decrease in speed of sound, following matrix degradation, suggests that it may be related to the function of the proteoglycan aggregate in articular cartilage, which acts to oppose the tensile forces of the collagen fibrils. Following matrix degradation and proteoglycan loss, this swelling force is removed, altering the poroelastic properties and softening the cartilage. The exact mechanism by which this results in a decreased speed of sound is not clear.

The frequency-dependent attenuation of normal human and bovine cartilage has previously been reported in the range of 20 to 40 MHz. Our results in human and bovine cartilage dem onstrated that, in this frequency range, experimentally-induced matrix degradation resulted in a 20 to 30% (p < 0.05) increase in the frequency-dependent attenuation. A previous in vivo study by Agemura et al. compared attenuation coefficients after depleting adult bovine cartilage proteoglycans. Contrary to our observations, their work, performed at 100 MHz, showed a decrease in the attenuation coefficient following proteoglycan depletion. Figure 4 presents the frequency-dependent attenuation for young normal, control and papain-digested human cartilage, and table 1 presents the best fits of our measurements with Eq. (1). The attenuation coefficient of the control samples, which were obtained from osteoarthritic patients during total knee arthroplasties, was consistently higher than the attenuation for young normal cartilage. Similarly, the papain-digested cartilage had a consistently higher attenuation than that of the control specimens. The observed increase between proteoglycan-depleted and control cartilage specimens was statistically significant (p < 0.05), as was the observed increase between young normal and control samples (p < 0.05) when verified with an unpaired Student's t-test. We suggest that the control human cartilage samples had a frequency-dependent attenuation higher than the young normal samples be-
0.04. A summary of the speed of sound data for the 12 paired control and proteoglycan-depleted bovine cartilage samples is presented in Figure 5.

The mean attenuation coefficient, measured at 30 MHz, was 30% higher in the 12 interleukin-1 cultured bovine cartilage samples than in the 12 corresponding control cartilage samples (9.1 ± 1.0 vs. 6.8 ± 1.2 dB/mm). The observed mean increase in frequency-dependent attenuation was statistically significant, \( p = 0.04 \). A summary of the frequency-dependent attenuation data for the 12 paired control and proteoglycan-depleted bovine cartilage samples is presented in Figure 6.

DISCUSSION

Our experiments in human and bovine articular cartilage show that matrix degradation and proteoglycan loss results in a decrease in the speed of sound and an increase in the frequency-dependent attenuation. We used two independent experimental models of osteoarthritis: digestion of human cartilage with papain and culture of bovine cartilage with interleukin-1. Both models function by primarily degrading the proteoglycans, although the mechanism of degradation for each method is different. Previous researchers have reported the speed of sound in normal human cartilage to be 1665 m/s. Our results of 1666 ± 16 m/s conform to these previously published values. The 2% (\( p < 0.05 \)) decrease observed in our speed of sound data for human and bovine cartilage, following matrix degradation and proteoglycan loss, is consistent with the observation of Myers. Myers also reported a 4% mean decrease in the speed of sound when comparing osteoarthritic and normal human cartilage specimens; however, they did not attempt to establish a significant correlation between the speed of sound in normal and osteoarthritic cartilage specimens. Conversely, the data available for bovine cartilage, measured at 100 MHz, indicates a slight increase in the speed of sound after depleting bovine cartilage of chondroitin sulfate, a glycosaminoglycan or collagen. The observed decrease in speed of sound, following matrix degradation, suggests that it may be related to the function of the proteoglycan aggregate in articular cartilage, which acts to oppose the tensile forces of the collagen fibrils. Following matrix degradation and proteoglycan loss, this swelling force is removed, altering the poroelastic properties and softening the cartilage. The exact mechanism by which this results in a decreased speed of sound is not clear.

The frequency-dependent attenuation of normal human and bovine cartilage has not previously been reported in the range of 20 to 40 MHz. Our results in human and bovine cartilage demonstrate that, in this frequency range, experimentally-induced matrix degradation resulted in a 20 to 30% (\( p < 0.05 \)) increase in frequency-dependent attenuation. A previous investigation by Akebura et al. compared attenuation coefficients after depleting adult bovine cartilage proteoglycans. Contrary to our observations, their work, performed at 100 MHz, showed a decrease in the attenuation coefficient following proteoglycan depletion. Figure 4 presents the frequency-dependent attenuation for young normal, control and papain-digested human cartilage, and Table I presents the best fits of our measurements with Eq. (1). The attenuation coefficient of the control samples, which were obtained from osteoarthritic patients during total knee arthroplasties, was consistently higher than that of the control specimens. The observed decrease between proteoglycan-depleted and control cartilage specimens was statistically significant (\( p < 0.05 \)), as was the observed decrease between young normal and control samples (\( p < 0.05 \)) when verified with an unpaired Student's t test. We suggest that the control human cartilage samples had a frequency-dependent attenuation higher than the young normal samples be-
TABLE 1. Frequency-dependent attenuation for young normal, control and papain-digested human cartilage fitted to Eq. (1). The exponent value represents the frequency-dependent term, $\gamma$.

<table>
<thead>
<tr>
<th></th>
<th>Young normal $\alpha_n(f) = 0.059 f^{1.37}$</th>
<th>Control $\alpha_c(f) = 0.112 f^{1.23}$</th>
<th>Proteoglycan-depleted $\alpha_{Pt}(f) = 0.216 f^{1.08}$</th>
</tr>
</thead>
</table>

cause they had already undergone some osteoarthritic changes prior to removal during knee arthroplasty. Hence, the control cartilage samples may have already been depleted of some matrix proteoglycans. Thus, these samples may represent an intermediate stage in proteoglycan loss between young normal cartilage and papain-digested articular cartilage. These observations suggest that the frequency-dependent attenuation is sensitive to the concentration of matrix proteoglycans in the articular cartilage.

The mean frequency-dependent attenuation results from the human and bovine articular cartilage specimens were plotted graphically in figures 4 and 6, respectively. The data on these graphs were then fitted with Eq. (1) to model the frequency dependence of the mean attenuation values. The attenuation mechanism is primarily comprised of scattering and absorption in the interactions of ultrasound with tissue. We suggest that the observed increase in the attenuation coefficient associated with matrix proteoglycan loss may arise from changes to the orientation of the collagen fibril network. Williams et al. observed a loss of matrix proteoglycans as well as an alteration in the spatial orientation of collagen following intra-articular papain injection in rabbits. Their observations showed that the collagen fibrils changed from an anisotropic, or dermal structure to a more random, isotropic organization. The collapse of the collagen fibrils was attributed to the loss of the ability of the proteoglycans to absorb the cartilage and oppose the tensile forces of the collagen fibrils. Previous investigations of the ultrasound characterization of cartilage have indicated a correlation between the orientation of collagen and the attenuation at 100 MHz. We suggest that the change in collagen orientation associated with matrix degradation and proteoglycan loss, from our experimental models of osteoarthritis, may contribute to the observed increase in the attenuation coefficient of ultrasound. Although we believe the increase in attenuation arises from the alteration in collagen orientation, it is not clear whether the increase arises from changes in scattering or absorption of the ultrasound.

CONCLUSIONS

In conclusion, the first measurements of speed of sound and attenuation of human and bovine cartilage at 37 °C have been performed in the 20 - 40 MHz range. Sound speeds in human and bovine cartilage in the 20 - 40 MHz range are consistent with previous measurements (~1,665 m/s). Experimentally-induced degradation of the matrix in human and bovine articular cartilage in creases the attenuation coefficient by 20 to 30% (p < 0.05) and decreases the speed of sound by 2% (p < 0.05) in the 20 - 40 MHz range. These changes are consistent in two independent models of matrix degradation in both human and bovine articular cartilage samples. The mean attenuation coefficient of un degraded human cartilage at 30 MHz was 6.2 ± 0.4 dB/mm. The ultrasonic properties of articular cartilage appear to be sensitive to the concentration of matrix constituents, such as proteoglycans and collagen. Although statistically-significant differences are observed between the sample groups tested, the variability
of the data precludes the use of such a test to determine matrix degradation in an individual subject. Further studies are needed to determine if the source of the variability is due to natural variation in the tissue itself or for experimental variables such as sample thickness that can be controlled. In any event, it may be possible to follow in individual in a relative sense if suitable minimally-invasive analysis approaches are developed. Such approaches could be implemented using an high frequency ultrasonic system in which the probing transducer is introduced to the joint space by a semi-flexible catheter. Because matrix degradation and proteoglycan loss occurs in osteoarthritis, we believe that ultrasound tissue characterization may potentially have a role in the assessment of articular cartilage during osteoarthritis progression.

REFERENCES


