Can proteoglycan change in articular cartilage be detected by ultrasound evaluation?

Afara, I., Wallace, S., Singh, S., and Oloyede, A.

School of Engineering Systems, Institute of Health and Biomedical Innovation, Faculty of Built Environment and Engineering, Queensland University of Technology, Brisbane, Australia.

Abstract. This paper assesses the capacity of high-frequency ultrasonic waves for detecting changes in the proteoglycan (PG) content of articular cartilage. 50 cartilage-on-bone samples were exposed to ultrasonic waves via an ultrasound transducer at a frequency of 20*MHz*. Histology and *ImageJ* processing were conducted to determine the PG content of the specimen. The ratios of the reflected signals from both the surface and the osteochondral junction (OCJ) were determined from the experimental data. The initial results show an inconsistency in the capacity of ultrasound to distinguish samples with severe proteoglycan loss (i.e. >90% PG loss) from the normal intact sample. This lack of clear distinction was also demonstrated at for samples with less than 60% depletion, while there is a clear differentiation between the normal intact sample and those with 55-70% PG loss.

Introduction

Several studies have investigated the responses of articular cartilage in different physiological conditions to ultrasound wave, including the effects of surface roughening [1], collagen disruption [2, 3], proteoglycan depletion [4, 5] and naturally degenerated or osteoarthritic conditions [5, 6]. A common limitation with practically all of these studies, which are based on the analysis of reflected signals from the cartilage – bone interface, is the variabilty in the pattern of the reflected signal. This inevitable and difficult to control influence is a consequence of the varying degrees of attenuation and diffraction properties of normal tissues [7] and the rough nonuniform topography of the osteochondral junction.

Another limitation of the literature material is that no study has related the ultrasound reflection patterns with quantity of proteoglycans (e.g. in a gradual

depletion process), the degree of disruption of the collagen fibrillar meshework or their combined altered osmotic state. This paper investigates the influence of the quantity of proteoglycans in the articfically degraded cartilage on the pattern of the reflected ultrasound signal relative to the ratio of the osteochondral junction to surface reflected wave proposed earlier by Brown et al [8].

Material and Methods

Sample Preparation

Visually normal and intact bovine patellae, (N=6), harvested from prime oxen within 24 h of slaughter, were used in this study. The samples were wrapped in 0.15M saline soaked towels and stored at about -20^{0}C until required for testing. Prior to testing, the intact patellae were thawed in 0.15M saline at room temperature for about 4 hours, then cartilage-on-bone blocks (n = 26, lxbxh = 7x7x5mm) were extracted from the patellae. All tests were conducted with the specimens fully submerged or hydrated in 0.15M saline.

Ultrasound echoes were taken from the saline-cartilage and cartilage-bone interfaces of all the samples. Care was taken to ensure that the tissue surface was parallel to the surface of the transducer. This is to make sure all the reflected ultrasound signal is captured by the transducer. Subsequent to ultrasound examination, the samples were treated in 1*mg/ml* of trypsin (T4667, Sigma Aldrich, Sydney, Australia) in 0.15M phosphate buffered saline at 37^{o}C to remove proteoglycans. The proteoglycan depletion programme was performed for 4hrs, with ultrasound reflection signals obtained from the samples at 1hr intervals. This gradual progression of enzymatic action from surface to bone closely resembles the pattern of proteoglycan loss in early stage osteoarthritis which is characterised by gradual loss of proteoglycans and disruption of the collagen meshwork from the superficial zone [9] amongst others signs.

Experimental Set-up

Ultrasound examinations were made at an approximate distance of 3mm from the sample surface using a 20MHz, **Ø**3*mm*, plane-ended contact transducer (V129, Panametric Inc., Massachusetts, USA). The transducer was excited via a pulser/receiver that also receives the reflected ultrasound echo. This was in turn connected to PC-based oscilloscope – PC 5204 (Pico technology Limited, Cambridgeshire, UK) which captures and converts the analog signal to digital in real-time and then displays it on a PC. Surface and osteochondral junction (OCJ) reflections were captured and recorded using the PicoScope 6 software (Pico technology Limited, Cambridgeshire, UK).

Histological Evaluation and Image Analysis

Sections of each sample was excised at intervals of 1hr for histological evaluation.

Subsequent to ultrasound examination, 7µm-thick sections were excised from the samples and fixed on microscopic slides. The sections were then stained with Safranin-O (which binds stoichiometrically with proteoglycans). This was followed by absorbance profiling under monochromatic light source using a Nikon Labo-Phot light microscope to obtain the micrographic images of the sections before and after staining.

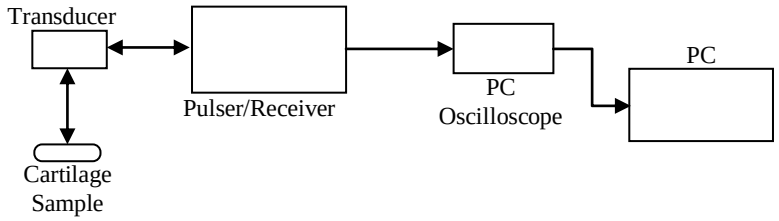

Figure 1. Experimental setup for ultrasonic evaluation of cartilage

Image analysis to determine the proteoglycan content was performed using *ImageJ* software version *1.44i* (Wayne Rasband, National Institute of Health, USA) according to protocols observed by Moody et al [10] and Brown et al [11]. In summary, the stained and unstained section micrographs of each sample are converted to an absorbance–depth profile. Since the light absorption is a consequence of the Safranin-O stained proteoglycans, the absorbance profile is directly related to the proteoglycan distribution and content of the sample. Using an ImageJ macro written in-house for this purpose, the proteoglycan content in the sample was calculated based on Beer Lambert's law. The proteoglycan content in the depleted sample was expressed as a percentage of the amount remaining in the tissue after depletion relative to the amount in its corresponding normal tissue.

Results

The patterns of reflections from the cartilage surface and the osteochondral junction (OCJ) between cartilage and bone were obtained for normal intact and progressively depleted samples (fig.2). The typical ImageJ results for the progressive removal of proteoglycans from the samples from 1 to 4 hr exposure for trypsin are presented in fig.3a. These data was used to estimate the percentage of proteoglycan that was removed from the various samples (Table I). The reflection ratio ranged from 2.67±1.52 for normal samples to 5.11±1.65 (mean±S.D) for proteoglycan depleted samples.

The results demonstrate that there is an apparent in consistency or discontinuity in the variation of the median values of the ratios for the surface to OCJ reflections where the ratio decreased from the normal to samples depleted for 2hrs, and then, rose for both the 3 and 4 hr enzymatically treated samples (fig. 3b,c).

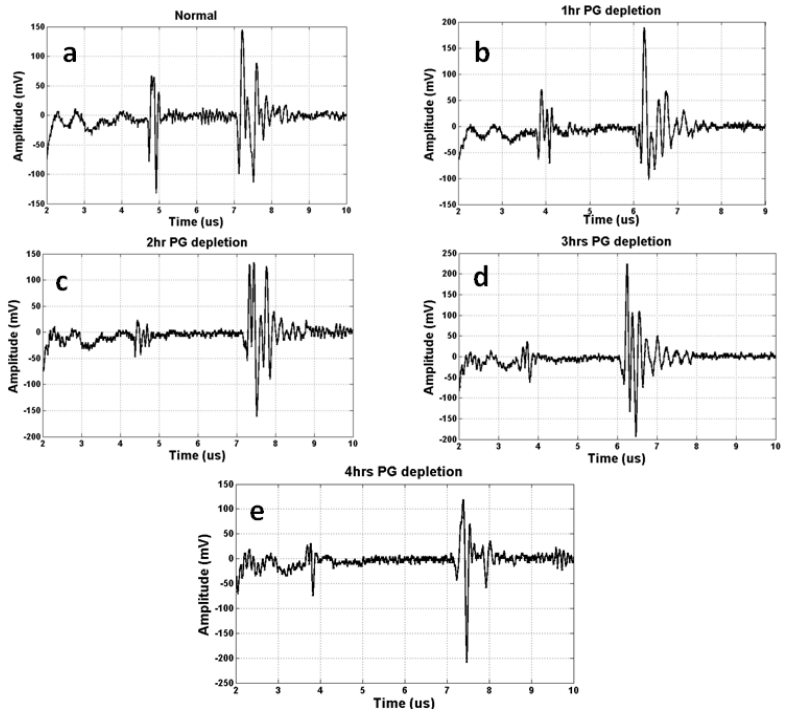

Figure 2. Typical ultrasound signal reflection profile for (a) normal; (b) 1hr PG depleted; (c) 2hrs PG depleted; (d) 3hrs PG depleted; and (e) 4hrs PG depleted articular cartilage showing surface reflection (first peak) and osteochondral junction reflection (second peak)

The ImageJ absorbance profile of (fig 3a) shows the distribution of proteoglycans from cartilage surface to bone for normal and depleted samples. The approximate proteoglycan content was obtained by calculating the area under the ImageJ absorbance curve (fig 3a).

Sample	**Ultrasound reflection ratio**	**Approximate % PG depleted**
Normal	0.357±0.208	0
1hr PG depleted	0.320±0.274	68.53
2hr PG depleted	0.226±0.129	70.42
3hr PG depleted	0.425±0.327	93.41
4hr PG depleted	0.389±0.287	100

Table I. Quantity of proteoglycan, as a percentage of total proteoglycan content of samples, removed by exposing cartilage samples to trypsin for 1 to 4 hrs, calculated as the area under the ImageJ curves in fig.3a.

Discussion and Conclusion

This study has re-assessed the capacity of ultrasound to differentiate between normal and proteoglycan depleted articular cartilage. However, unlike the single proteoglycan depletion protocol, e.g. exposure of cartilage to a single known duration and comparing the resulting specimen to the normal, this present investigation investigates the ultrasound reflection from samples that had been subjected to progressive proteoglycan removal for 1 to 4 hrs. This provides a more comprehensive assessment of the capacity and limitation of ultrasound-based evaluation of the integrity of the cartilage matrix.

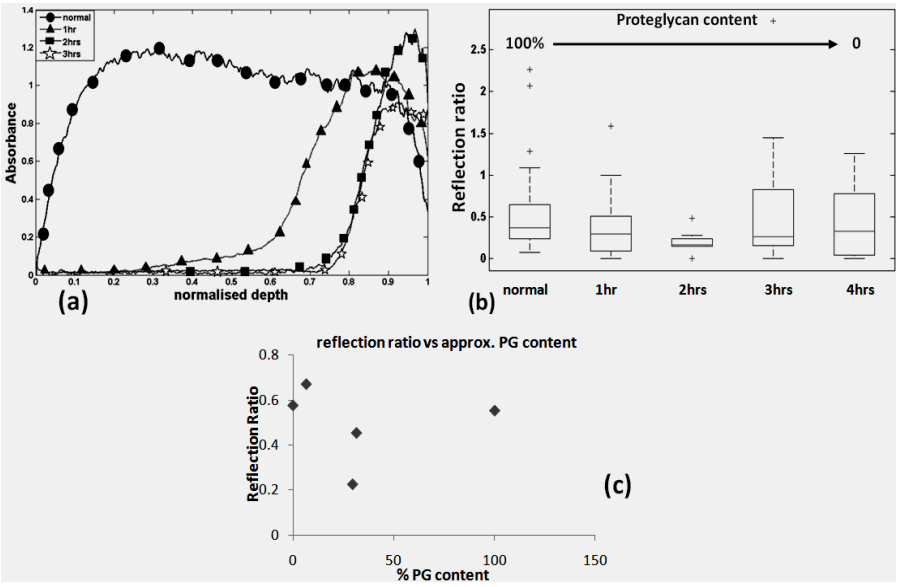

Figure 3. (a) ImageJ absorbance profile for normal and depleted cartilage samples. (b) Boxplot of normal and proteoglycan (PG)-depleted samples. normal; 1hr group: sample with approx. 68.53% PG depletion; 2hrs group: sample with approx. 70.42% PG depletion; 3hrs group: sample with approx 93.41% PG depletion. (c) %Proteoglycan content versus reflection ratio

These current results (fig. 3b and c) raise the question of the ability of the ratio of the surface-to-OCJ reflections to consistently distinguish samples with severe proteoglycan loss (i.e. >90% PG loss) from normal intact ones (Fig. 4). This lack of clear distinction was also demonstrated at <50% depletion, while there is a clear differentiation between the normal, and samples with between 55-70% of their proteoglycans removed. It should be noted that most reported data are on the

comparison of cartilage that had been exposed to enzyme for about 2hrs (i.e. 55–70%) to normal specimens.

It is difficult at this stage to determine fully the reason for this inconsistency. It may be due to a limitation in the capacity of the reflection ratio for cartilage evaluation as argued above or certain issues around the enzymatic digestion of the proteoglycans. For example, in order to preserve the altered cartilage matrix and restrict the modification to that due to the enzymatic digestion only, we used a higher concentration of trypsin relative to the quantity used in for example Brown et al [8]; it probable that this type of departure from the method previously reported has an effect on our current results.

In conclusion, the reported results in this paper suggest that high frequency ultrasound method can distinguish between normal intact and degenerated articular cartilage. However, it seems that a better analysis leading to a different parameter of assessment beyond the surface-to-OCJ reflection ratio is required for consistent evaluation cartilage health.

Reference

[1] Saarakkala, S., Töyräs, J., Hirvonen, J., Laasanen, M.S., Lappalainen, R., and Jurvelin, J.S., (2004), *Ultrasound Med Biol*, vol. 30, p. 783–792.
[2] Hattori, K., Mori, K., Habata, T., Takakura, Y., and Ikeuchi, K., (2003), *Clinical Biomechanics*, vol. 18, p. 553-557.
[3] Nieminen, H.J., Töyräs, J., Rieppo, J., Nieminen, M.T., Hirvonen, J., Korhonen, R., and Jurvelin, J.S., (2002), *Ultrasound Med Biol*, vol. 28, p. 519–525.
[4] Pellaumail, B., Watrin, A., Loeuille, D., Netter, P., Berger, G., Laugier, P., and Saïed, A., (2002), *Osteoarthritis Cartilage*, vol. 10, p. 535–541.
[5] Brown, C.P., Hughes, S., Crawford, R.W., and Oloyede, A., (2007), *Connective Tissue Research*, vol. 48, p. 277-285.
[6] Chérin, E., Saïed, A., Laugier, P., Netter, P., and Berger, G., (1998), *Ultrasound Med Biol*, vol. 24, p. 341–354.
[7] Senzig, D.A., Forster, F.K., and Olerud, J.E., (1992), *Journal of the Acoustic Society of America*, vol. 92, no. 2, p. 676-681.
[8] Brown, C.P., Hughes, S., Crawford, R.W., and Oloyede, A., (2008), *Phys. Med. Biol.*, vol. 53, no.15, p. 4123.
[9] Carney, S.L., Billingham, M.E.J., Muir, H., and Sandy, J.D., (1984), *Journal of Orthopaedic Research*, vol. 2, p. 201-206.
[10] Moody, H.R., Brown, C.P., Bowden, J.C., McElwain, D.L.S., Crawford, R.W., and Oloyede, A., (2006), *Journal of Anatomy*, vol. 209, p. 259-267.
[11] Brown, C.P., Moody, H.R., Crawford, R.W., and Oloyede, A., (2007), *Connective Tissue Research*, vol. 48, p. 52-61.