Measurement of the layered compressive properties of trypsin-treated articular cartilage: an ultrasound investigation

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Abstract—*An ultrasound-compression system has been developed for the study of the layered biomechanical properties of articular cartilage. Cartilage specimens harvested from the bovine patella groove, with and without trypsin digestion, were tested using this system. It was noted that a large ultrasound reflection can be detected in the interface of the trypsin digestion front. This ultrasound reflection signal was used to differentiate the deformations of different portions of the cartilage throughout its depth when a load was applied. The equilibrium compression moduli of the digested, undigested and entire portions of articular cartilage were measured. The modulus of the cartilage without any digestion was 660 ± 230 kPa. After 1 h digestion with 1 mg ml^{-1} trypsin solution, the thickness of the digested portion was 0.50 ± 0.06 mm, and the modulus of the entire cartilage layer changed to 125 ± 42 kPa. The moduli of the digested and undigested portions were 58 ± 24 kPa and 470 ± 31 kPa, respectively. Similar results were obtained for the cartilage with trypsin digestion for 2 h.*

Keywords—*Articular cartilage, Biomechanics, Elastography, Osteoarthritis, Ultrasound technique*

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1 Introduction

ARTICULAR CARTILAGE (AC) is a biological weight-bearing tissue covering the ends of articulating bones within synovial joints. Functionally, articular cartilage plays essential roles in joint lubrication and in load transmission across joints (KEMPSON, 1980; MOW *et al.*, 1991). Structurally, the organic composite matrix of AC can be regarded as a proteoglycan gel reinforced by a network of fine collagen fibrils and swollen with a multi-ionic electrolytic aqueous solution (MUIR, 1980; MOW *et al.*, 1991). Degeneration of this tissue under pathological situations such as osteoarthritis (OA) can lead to significant compromises in joint functions and thus to patient disabilities (MANKIN *et al.*, 1994).

The cartilage changes during OA include loss of proteoglycan, surface fibrillation and increasing hydration, leading to compromises of the compositional and biomechanical properties. One of the important pathological processes that compromise the biomechanical properties of articular cartilage in the early stage of OA involves the structural changes in the proteoglycan aggregates caused by endogenous proteolytic enzymes (BUCKWALTER and MANKIN 1997). Although biochemical (BRANDT, 1974), biomechanical (ARMSTRONG and MOW, 1982) and electromechanical (BERKENBLIT *et al.*, 1994) methods have been applied to investigate the early degeneration of articular cartilage during OA, it is still difficult to measure the degree of cartilage degeneration in a non-destructive manner.

In the early stage of degeneration, the depletion of proteoglycan and the increase in water content can occur primarily in the top layer of the tissue near the articular surface (BUCKWALTER and MANKIN 1997). To detect such an early sign, it is important to measure the inhomogeneous changes in the biomechanical properties. The zonal variations in the biomechanical properties of AC have been measured in tension using carefully excised tissue slides (WOO *et al.*, 1976; ROTH and MOW, 1980; DROGENDIJK and MOW, 1982; GUILAK *et al.*, 1994). However, similar studies have rarely been reported for the inhomogeneous compressive properties of AC.

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Confined compression has been carried out with and without the superficial zone to assess its influence on the biomechanical properties of cartilage under compressive loading (SETTON *et al.*, 1993). A method to quantify the inhomogeneous equilibrium strain within AC during confined compression has been reported (SCHINAGL *et al.*, 1996; 1997). A video microscope with an image-processing technique was employed to derive the strain fields along one side of the excised slide of AC. The fluorescently labelled chondrocyte nuclei were selected as the intrinsic markers. However, given its *in vitro* and invasive nature, this technique seems difficult to improve for *in vivo* testing and clinical diagnosis.

Ultrasonic characterisation of AC has been the subject of many recent investigations owing to its non-destructive manner. In addition, the acoustic, structural and mechanical properties of materials are related to each other; it is believed that ultrasound may provide a sensitive technique for quantitative analysis of early OA.

Many studies have reported on the suitability of ultrasound for the measurement of AC thickness (RUSHFELDT *et al.*, 1981; MODEST *et al.*, 1989; JURVELIN *et al.*, 1995; MYERS *et al.*, 1995; ADAM *et al.*, 1998). TEPIC *et al.*, (1983) measured the changes in AC thickness and acoustic impedance induced by altering the osmotic pressure. A model was proposed to relate the measured results to the cartilage microstructure. Other acoustic parameters, such as ultrasonic speed (MYERS *et al.*, 1995; AGEMURA *et al.*, 1990), attenuation (SENZIG *et al.*, 1992; TOYRAS *et al.*, 1999), echo pattern (MYERS *et al.*, 1995; KIM *et al.*, 1995; SAIED *et al.*, 1997) and reflection coefficient (ADLER *et al.*, 1992 CHERIN *et al.*, 1998; TOYRAS *et al.*, 1999), have been used for characterisation of AC in healthy and OA conditions.

It can be claimed that, as the functions of AC are clearly biomechanical, it is extremely relevant to the early diagnosis of AC to develop an ultrasound assessment procedure that can determine the changes in the biomechanical properties of AC at different depths. The idea of imaging soft-tissue mechanical properties using ultrasound with an applied load on the tissues was first introduced by OPHIR *et al.* (1991). In this elastography method, local tissue displacements were estimated from the time delays between the pre- and post-compression ultrasonic echo signals by using a cross-correlation technique. The axial gradients of the displacements could then be calculated for estimation of the local strains. Recently, research has been undertaken into the applications of this imaging technique (KALLEL *et al.*, 1998), improvement of the strain estimation algorithm (SKOVORODA *et al.*, 1999; BAI *et al.*, 1999) and extending the elasticity imaging to microscopic level (COHN *et al.*, 1997*a*; *b*).

As a relative measurement of material property is sufficient for imaging purposes, the absolute value of a tissue material parameter, such as Young's modulus, was generally not addressed in this new imaging modality. On the other hand, we developed an ultrasound palpation system with a pen-size test probe for the measurement of the mechanical properties of soft-tissue layers (ZHENG and MAK, 1996; 1999). The ultrasound technique was used to measure thickness changes in a soft-tissue layer upon load application. Biomechanical models were used to extract material parameters of the tissues from load-indentation responses.

This ultrasonic approach to the measurement of tissue elastic properties has recently been introduced into the biomechanical study of AC for compression properties (ZHENG *et al.*, 1998), indentation properties (SUH *et al.*, 1999) and dynamic properties of Poisson's ratio (FORTIN *et al.*, 2000). No study has been reported on the ultrasonic measurement of the elastic properties of different AC layers through the depth of the AC. One of the challenges is to compress the cartilage specimen while retaining the high resolution of the ultrasonic measurement with a high signal-to-noise ratio.

Cartilage has a relatively uniform structure in comparison with other soft tissues, such as muscles (MUIR, 1980; MOW *et al.*, 1991). Thus, the ultrasound backscattering signals from cartilage are relatively weak. In addition, passing the ultrasound beam through a compressor during a compression test will further weaken the signal-to-noise ratio of the echo signals. For this reason, in the elasticity microscope presented by COHN *et al.* (1997*a*, *b*), the ultrasound beam was made to pass through a slit (2.6 mm in width) in the compressor. However, successful biomechanical interpretation of the load-deformation responses of AC also depends on a well-defined boundary condition. This slit could introduce uncertainties into the mechanical boundary conditions of the sample when used for biomechanical studies of AC (FORTIN *et al.*, 2000).

The purpose of this paper was to report a study of the effects of trypsin digestion on bovine patella AC using an ultrasound-compression method. Experimentally controlled trypsin digestion was used to deplete the proteoglycan aggregates of AC to various depths (LEUNG *et al.*, 1998; LYYRA *et al.*, 1999; TOYRAS *et al.*, 1999). The equilibrium compression moduli of the digested and undigested portions of full thickness AC were measured in this study.

2 Materials and methods

2.1 *Specimen preparation and histology study*

Five pieces of fresh mature bovine patella without obvious lesion were obtained from a local slaughterhouse within 6 h after slaughter. The patellae were stored at under −20°C until the experiments. During specimen preparation, the patellae were first thawed in normal saline solution (0.15 M NaCl) at room temperature, 20°C, for 1 h, and three osteochondral cylinders were then cut out from the flat area of each patella using a metal punch with a diameter of 10.7 mm. Two cartilage cylinders from each patella were randomly selected and immersed in trypsin solution (normal saline solution with 1 mg ml^{-1} trypsin type III from bovine pancreas*) for 1 and 2 h, respectively, and the third was stored in normal saline solution as a control.

A preliminary histology study of AC using safrannin-O stain was carried out, showing the extent of digestion (LEUNG *et al.*, 1998). The intensity of the safrannin-O stain (red in a colour picture) represented the amount of proteoglycan aggregates. The proteoglycans were depleted in the digested portion of the AC specimen.

As shown in Fig. 1, a distinct digestion front, indicated by the arrows, could be noted in specimens after 1 h of trypsin digestion. The image shows the central part of the disc specimen. The digestion front apparently advanced towards the cartilage–bone interface with further digestion (LEUNG *et al.*, 1998). The penetration depth of the trypsin digestion measured using the histology study was correlated with that measured using ultrasound in this study.

After trypsin digestion, the bone portions of all 15 cartilage plugs were cut to approximately 1 mm thick using an ISOMET lower-speed saw†. A smaller punch, with a diameter of 7.9 mm, was used to obtain a smaller cartilage disc from the central portion of each cartilage-bone disc to remove the digested sides of the cartilage. All the re-punched cartilage disc, with or without digestion, were then bathed in normal saline solution with enzyme inhibitors (2 mM ethylenediaminetetra-acetic acid (EDTA), 5 mM benzamidine-hydrochloride, 10 mM N-ethylmaleimide (NEM) and 1 mM phenylmethylsulfonyl fluoride

* Sigma, St Louis, MO, USA
† Buehler Ltd, Lake Bluff, IL, USA

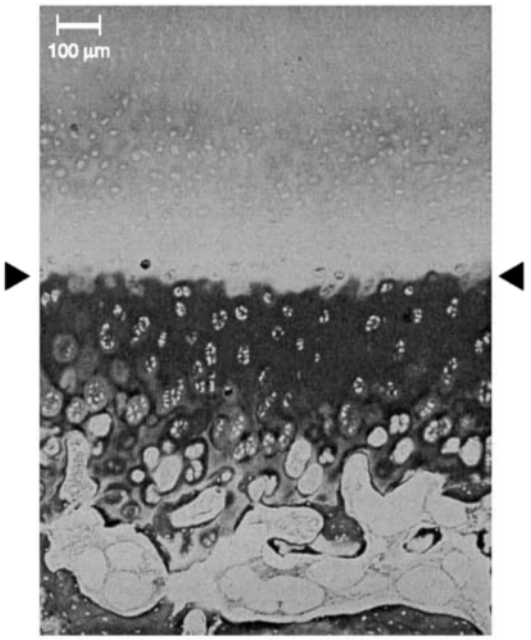

Fig. 1 *Typical histological image of central portion of trypsin-digested AC with safrannin-O stain (red in colour image)*

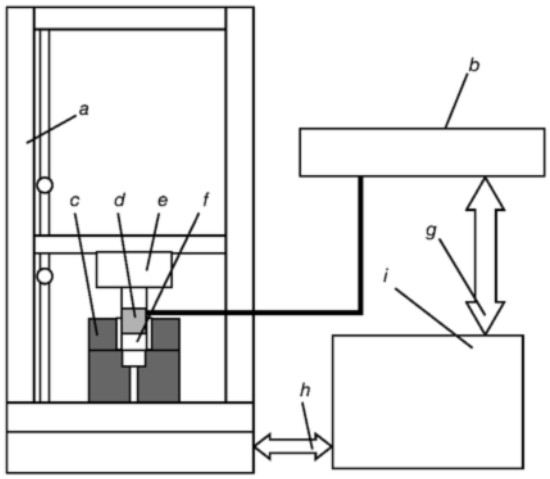

Fig. 2 *Diagram of ultrasound-compression system for articular cartilage: (a) material testing machine; (b) ultrasound pulser/receiver; (c) container for cartilage specimen; (d) 50 MHz contact ultrasound transmitter/receiver; (e) load cell of material testing machine; (f) cartilage specimen; (g) 500 MHz A/D converter card; (h) RS232 interface; (i) Pentium 200 MHz personal computer.*

(PMSF)) for 1 h, and, finally, stored at −20°C until the ultrasound-compression test.

2.2 *Ultrasound-compression system*

Fig. 2 shows a schematic diagram of the ultrasound-compression testing system used in this study. A testing chamber with a flat, rigid and impermeable bottom interface was mounted on the base of a Hounsfield material testing machine (model H10 KM/03) for installation of the cartilage specimen. A 50 MHz ultrasound transducer, with a diameter of 8 mm and a permanent 2.5 μs silica delay line‡, was used to transmit ultrasound pulses into the cartilage, to receive ultrasound reflections from interfaces within the cartilage and, also, to serve as an impermeable compressor. The interstitial fluids of the cartilage specimen could only flow in and out of the sides during compression and force-relaxation.

An ultrasound pulser/receiver** was used to drive the ultrasound transducer and to amplify the received ultrasound reflections. The ultrasound reflection signals were digitised by an A/D converter card with a sampling rate of 500 MHz†† installed in a Pentium 200 MHz PC. The movement of the ultrasound transducer was driven by the Hounsfield machine, which was controlled by a custom-developed computer program. The program was also used for collecting ultrasound signals digitised by the A/D converter, reading the load and displacement data transferred from the material testing machine via the RS232 and subsequent signal processing. To improve the ultrasonic signal condition for the later cross-correlation algorithm, synchronised ultrasound echo trains were averaged 1000 times to enhance the signal-to-noise ratio. To minimise the influence of the high-amplitude reflected signal generated at the end of the delay line of the transducer, the ultrasonic signal was first recorded with saline solution only. This recorded signal was subtracted from the reflection signals later obtained with AC specimens.

‡Model V214BC, Panametrics, Waltham, MA, USA
**Model 5601A, Panametrics, Waltham, MA, USA
††Model CompuScope 8500PCI, Gage, Canada

The cross-correlation technique is generally used for the study of the similarity between two signals. The normalised correlation coefficient of two series of discrete values

$$X = \{x(0), x(1), \ldots, x(N-1)\}$$
$$Y = \{y(0), y(1), \ldots, y(N-1)\}$$

can be written as

$$R = \frac{\sum_{i=0}^{N-1}[x(i) - \bar{X}][y(i) - \bar{Y}]}{\sqrt{\sum_{j=0}^{N-1}[x(j) - \bar{X}]^2 \sum_{k=0}^{N-1}[y(k) - \bar{Y}]^2}} \quad (1)$$

where $\bar{X}$ is the mean of X, and $\bar{y}$ is the mean of Y. The normalised correlation coefficient R indicates the similarity between the two signals. If they are exactly the same, then $R = 1$, and, if they have no similarity, then $R = 0$. $R = -1$ indicates that the two signals are exactly inverted about the amplitude.

If X and Y are two segments of ultrasonic signals that have been digitised, Eqn 1 can be used to determine the similarity between the two ultrasound reflections. Assuming that a specific interface has a unique pattern in ultrasound echo trains, the motion of the interface can then be tracked by the monitoring of the specific pattern in different ultrasonic echo trains after a certain load is applied to AC. The process can be achieved by shifting the specific echo in one echo train with respect to another echo train until the echoes overlap. This corresponds to searching for the maximum in the normalised correlation coefficient and effectively measuring the difference in the arrival times of the specific echo that is reflected from a specific interface.

Fig. 3 shows the result of a typical searching process for the maximum correlation coefficient R. It represents that the selected segment of the ultrasound signals has shifted by six time points. The interval between two contiguous time points is 2 ns in this study, with a 500 MHz A/D converting rate. For the average ultrasound velocity of 1760 m s^{-1} (MODEST *et al.*, 1989) for AC used in this study, the displacement resolution was 1.76 μm. Hence, the displacement of the interface according to the time shift shown in Fig. 3 was $1.76 * 6\,\mu m = 10\,\mu m$. To

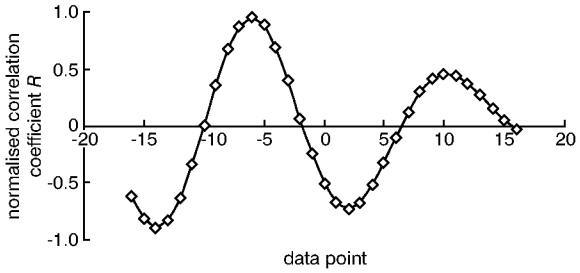

Fig. 3 *Result of typical searching process for maximum correlation coefficient*

improve the displacement resolution, an interpolation algorithm based on the time-shift property of a Fourier transform was applied (DING and BAI, 1998). The theoretical displacement resolution was improved to approximately 0.2 μm with tenfold interpolation.

The cross-correlation method has been used for ultrasound elastography of soft tissues (OPHIR *et al.*, 1991). In this study, it was used to track the movement of the interface between digested and undigested portions of AC layers when a deformation was applied to the surface.

2.3 *Ultrasound-compression test*

Before the ultrasound-compression test of an AC disc, it was first thawed in normal saline solution for 1 h at a room temperature of approximately 20°C. The tests were performed with the specimens bathed in the normal saline solution with enzyme inhibitors. During a test, the compressor, i.e. the ultrasound transducer, was first pushed gradually against the surface of the AC disc to make contact. The AC disc was then compressed at a rate of 0.1 mm min^{-1} until the stress reached 20 kPa. It was noted that the stress subsequently relaxed and equilibrated almost completely, with a stress changing rate of less than 0.5% min^{-1} after a period of 1700 s. This reference state was defined as the initial state. The ultrasound signals at this initial state were recorded. The ultrasound-compression test of each AC specimen was performed with a four-step compression procedure, as shown in Fig. 4. Each 0.01 mm compression was carried out at a compression rate of 0.1 mm min^{-1}. Ultrasound signals were recorded at 1700 ± 10 s after each compression step. Fig. 5 shows typical compression–relaxation behaviour of an AC disc specimen in response to the four steps of compression.

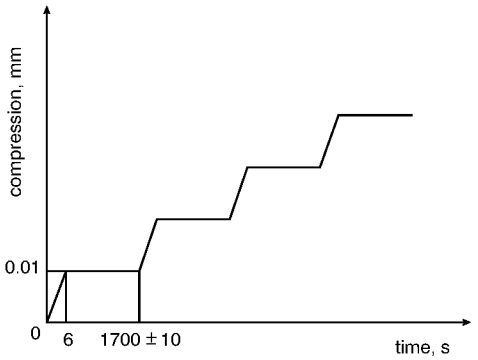

Fig. 4 *Compression scheme for ultrasound-compression test of AC. Four steps of compression of 0.01 mm each are applied. Ramp up period is 6 s, and relaxation behaviour is monitored for about 1700 ± 10 s for each compression step*

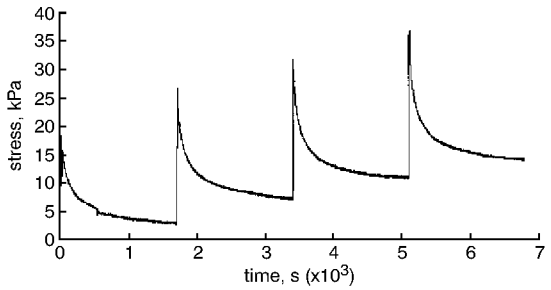

Fig. 5 *Typical compression–relaxation behaviour of AC specimen recorded during ultrasound-compression test*

2.4 *Calculation of modulus*

Figs 6*a–c* shows ultrasound signals recorded (*a*) from an AC specimen without trypsin digestion, (*b*) from an AC specimen with 1 h trypsin digestion, and (*c*) from the AC specimen, as (*b*), but after one step compression. Fig. 6*d* shows the intensity of ultrasound signal (*c*). The white, black and grey triangles mark the interfaces between the ultrasound transducer and the AC, between the digested and the undigested AC layers, and between the AC and the bottom of the container. The time measurement among these interfaces was used to calculate the thickness of the entire, the digested and the undigested layers of cartilage by assuming an ultrasound speed of 1760 m s^{-1} in AC (MODEST *et al.*, 1989). The effects of the zonal variation in ultrasound speed, particularly the difference between the ultrasound speed in the digested and the undigested zones, will be discussed later. The displacement of the interfaces after each step of the series compression procedures, as shown in Figs 4 and 5, were measured by the cross-correlation method, as described above.

In this study, the cartilage was compressed by approximately 1% during each step of the compression. It was assumed that the cartilage could slip to the sides freely with the lubrication of the saline solution during such a small compression. Hence, the uniaxial stress model was used to calculate the equilibrium compression modulus after each step of the compression and relaxation.

$$E = \frac{\sigma}{\varepsilon} = \frac{P/A}{d/h} \tag{2}$$

where E is the equilibrium Young's modulus, σ is the stress, ε is the strain, P is the applied load, d is the deformation of an AC

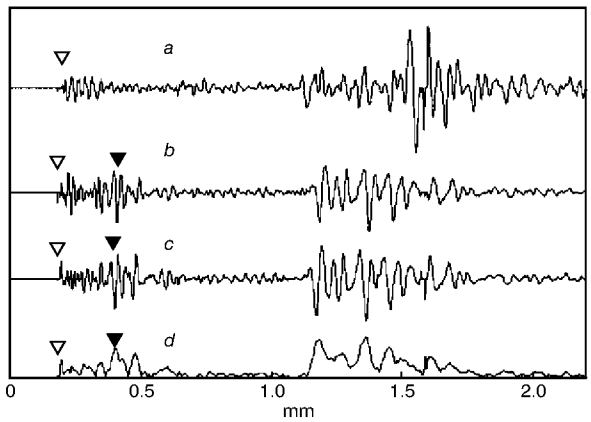

Fig. 6 *Typical set of ultrasound echo signals: (a) signal obtained before trypsin digestion; (b) signal obtained after 1 h trypsin digestion; (c) signal obtained after one step compression next to signal (b); (d) signal intensity of signal (c) with white, black and grey triangles representing interfaces between ultrasound transducer and cartilage, digested and undigested layers, and cartilage and bone, respectively*

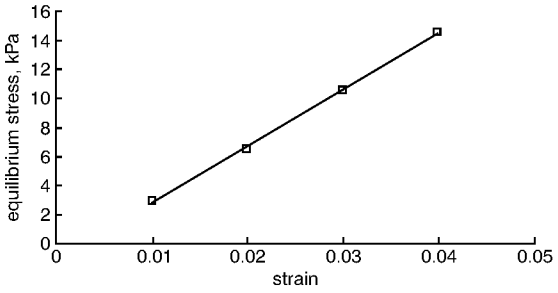

Fig. 7 *Calculation for equilibrium compression modulus using a linear regression of four equilibrium data points of stress and strain*

layer, h is the thickness of the AC layer, and A is the area of the AC disk. The ratio of σ/ε was determined by the slope of the linear regression of the four equilibrium stress-strain data points shown in Fig. 7. The moduli of the digested, the undigested and the entire layer of AC specimens were calculated in the same way as described above.

2.5 *Statistical analysis*

The changes in the Young's moduli, digestion-thickness AC specimens were analysed by paired t-tests. The correlation between the thicknesses of the digested AC portion measured with ultrasound and histology was analysed by two-factor ANOVA.

3 Results

As shown in Fig. 6*a* for a typical, undigested AC specimen, the ultrasound echo signals were quite small in the region with the cartilage. Much larger ultrasound echo signals were observed in a certain region, as shown in Fig. 6*b*, after the cartilage was treated with trypsin for 1 h. Similar findings were observed for the specimens with 2 h trypsin digestion. The cartilage specimens corresponding to the ultrasound signals shown in Figs 6*a* and *b* were not paired for obvious logistical reasons. As the main difference between the two specimens was that one was not digested and the other one was digested, it was reasonable to assume that the large ultrasound echo signals between the cartilage surface and bottom were from the interface of the digested/undigested portions of the AC specimen.

The thickness of the digested and undigested portions of the AC specimens obtained using the ultrasound measurement and the previous histological study (LEUNG *et al.*, 1998) is presented in Fig. 8. It was noted that the digestion front penetrated deeper into the AC layer as the digestion time increased; however, the penetration depth was apparently not proportional to the digestion time. The thicknesses of the digested portion measured with ultrasound and histology correlated quite well, as shown in Fig. 8. There was no significant difference ($p > 0.05$) between the results obtained with the two methods.

The elastic properties of the entire layer of the cartilage solid matrix decreased greatly after trypsin digestion. The equilibrium compression modulus of undigested AC specimens was 660 ± 230 kPa (standard deviation). It was calculated for the five cartilage specimens that were not digested and for the ten specimens before trypsin digestion. The equilibrium compression moduli of the specimens with 1 h and 2 h of trypsin treatment were 125 ± 42 kPa and 123 ± 53 kPa, respectively. They were calculated for the five digested cartilage specimens for each group. There was no significant difference ($p > 0.05$) between the compressive moduli of the specimens digested for

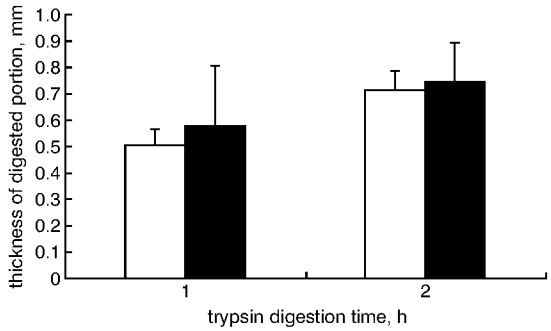

Fig. 8 *Relationship between thicknesses of digested portion and trypsin digestion time obtained using (□) ultrasound and (■) histology. Digested thickness was averaged from five specimens of each group for ultrasound measurement and from 18 specimens for histology measurement. Error bars represent standard more deviations*

1 h and those of the specimens digested for 2 h. The reason for this phenomenon may be that the digested portion was so 'soft' that its mechanical properties dominated the whole cartilage layer when a small deformation was applied. Therefore it was difficult to differentiate the extent of digestion using this traditional biomechanical test.

Fig. 9 shows the equilibrium compression modulus of digested and undigested portions of AC specimens as a function of digestion time. The elastic properties of the cartilage solid matrix of the digested portions greatly decreased. It was approximately ten times smaller compared with that of the undigested portions and specimens without digestion. The modulus of digested portions of AC layers with 2 h digestion (44 ± 10 kPa) was slightly, but significantly ($p < 0.05$), lower than that with 1 h digestion (58 ± 24 kPa).

Fig. 10 shows both the equilibrium compression modulus and the digestion penetration depth of the AC specimens with 1 h and 2 h trypsin treatment. Table 1 summarises the results of the equilibrium compression moduli of the digested zone, the undigested zone and the entire AC layer. It was noted that the modulus of the 'undigested' AC portions (after 1 h or 2 h trypsin digestion) was significantly ($p < 0.05$) smaller than that of the entire undigested AC layer. The possible reasons for this phenomenon will be discussed later.

4 Discussion

An ultrasound-compression testing system for the biomechanical assessment of trypsin-digested articular cartilage was introduced in this paper. The equilibrium compressive moduli

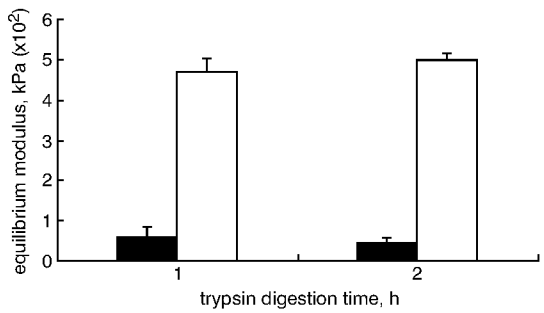

Fig. 9 *Equilibrium compression moduli of digested and undigested portions of AC specimens with 1 h and 2 h trypsin digestion. Moduli are average for five digested specimens for each group. Error bars represent standard deviations*

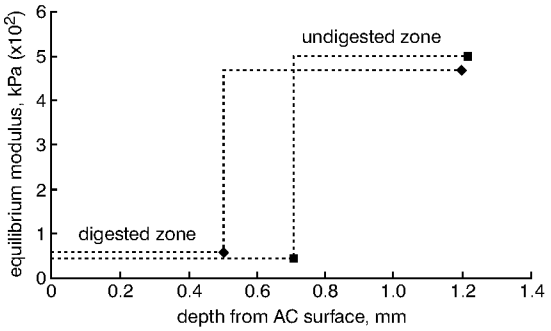

Fig. 10 *Change in measured equilibrium compression modulus and digestion penetration depth of AC specimens with (◆) 1 h and (■) 2 h trypsin treatment*

Table 1 Summary of measured equilibrium compression modulus of bovine patella cartilage

Digestion time, h	0		1		2	
Modulus, kPa	mean	SD	mean	SD	mean	SD
Entire layer	660	230	125	42	123	53
Undigested layer	N/A		470	31	500	160
Digested layer	N/A		58	24	44	10

of digested and undigested portions of cartilage were measured simultaneously without the cartilage being sliced. A contact ultrasound transducer with delay line was used to probe the interface of the trypsin digestion front and, meanwhile, served as a compressor. The displacement of the digested/undigested cartilage interface was measured by tracking the ultrasound echo reflected from that interface.

In this study, a constant ultrasound speed of 1760 m s^{-1} (MODEST *et al.*, 1989) was assumed for digested and undigested cartilage portions. The feasibility of using ultrasound for the measurement of the AC thickness has been discussed recently (MANN *et al.*, 1999; 2001). The main concern was that the ultrasound speed of AC was not a constant at different depths, at different locations or of different species. Some earlier studies had not demonstrated a significant difference between ultrasound speed in proteoglycan-depleted and intact cartilage (AGEMURA *et al.*, 1990; TOYRAS *et al.*, 1999); however, some studies had (MYERS *et al.*, 1995; SUH *et al.*, 1999). Further investigations are required to document the change in ultrasound speed in cartilage under simulated or real pathological conditions. The difference, if substantiated, would affect the thickness calculation. However, for the strain and modulus calculations in this study, the ultrasound speed was cancelled out. Thus the strain and modulus calculations were not affected by the zonal difference in ultrasound speed.

It was demonstrated that the equilibrium compression modulus of the digested AC portion was approximately ten times smaller than that of the undigested portion. This zonal variation in the biomechanical properties of AC could not be readily obtained with traditional biomechanical tests; apart from slicing the specimen (WOO *et al.*, 1976; ROTH and MOW, 1980; DROGENDIJK and MOW, 1982; GUILAK *et al.*, 1994). It has to be noted that the difference between the moduli in the digested and undigested regions of AC demonstrated in this study may also incorporate the effect of the depth-dependent mechanical properties of the intact AC (SCHINAGL *et al.*, 1997), in addition to the trypsin treatment. The equilibrium compressive modulus of AC reported in the literature ranged from several hundred kPa to several Mpa, depending on species, locations and pathological conditions (ATHANASIOU *et al.*, 1989; JURVELIN *et al.*, 1995; SCHINAGL *et al.*, 1997). The compressive modulus of AC also depended on the value of the assumed Poisson's ratio, whether it was measured using indentation or confined test (JURVELIN *et al.*, 1990).

It was noted that the modulus of the entire undigested AC layer (660 kPa) was significantly larger than that of the 'undigested' AC portions (470 kPa and 500 kPa after 1 h and 2 h digestion, respectively). This finding apparently contradicted the recent measurement, using a video technique, that the deep layer of AC was stiffer than the superficial and middle layers (SCHINAGL *et al.*, 1997). One reason for this phenomenon may be the non-linear properties of AC. For a given deformation applied to the surface of the AC, the strain in the 'undigested' portion was much smaller than that in the digested portion as well as the averaged strain in the undigested AC specimen. Owing to the non-linear properties of AC, the modulus determined for the undigested portion of AC was smaller than that of the entire undigested AC. Another possible reason may be that the digestion from the sides of the AC had not been fully removed. This issue needs to be clarified further.

The results of this paper and a recent study (TOYRAS *et al.*, 1999) demonstrated the feasibility of using the ultrasound method to track the advancing front of enzyme digestion; whether this approach would work equally well for degenerated tissue still remains to be validated. The thickness of the digested AC portion measured with ultrasound in this study correlated quite well with that measured with histology in a previous study. However, the specimens used in these two studies were not paired. Further paired correlation studies between the results measured with ultrasound and histology are required to investigate the accuracy of the thickness measurement using the ultrasonic signals. One important criterion for the success of the ultrasonic method was the presence of a distinct front of degeneration, with significantly different properties before and after the front. Although this was true for trypsin digestion, as demonstrated in this study, the same may not always be true in cartilage during the early phase of OA. Further validation and improvement are still needed. In addition, if the ultrasound beam was not perpendicular to the interface, the amplitude of the reflecting signal would be reduced. The effect of misalignment on the reflection amplitude was investigated in a previous study on skin and subcutaneous tissues using an ultrasound transducer of a similar diameter but with a lower frequency of 5 MHz (ZHENG *et al.*, 1999). It was demonstrated that the amplitude of the ultrasound reflecting signals could be reduced by 6% and 25% for misalignments of 2.5 and 5 degrees, respectively. The time shift of the ultrasound reflection was 2%, with a misalignment up to 10 degrees.

The ultrasound-compression method described in this paper provided a unique modality to measure the layered material properties of articular cartilage without damaging its biomechanical integrity. If the sensitivity of the ultrasound to weak acoustic interfaces can be further improved, or the presence of the distinct acoustic interface between the degenerated superficial layer and the deeper layer is validated, the method introduced in this study could be used for the diagnosis of OA following the development of a probe used with arthroscopes (LYYRA *et al.*, 1999). The mechanical properties of the degenerated superficial portion and the intact deeper portion can be measured individually using the technique introduced in this paper. Such information could not be obtained using traditional mechanical testing on the entire AC layer.

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