Altered Osmotic Swelling Behavior of Proteoglycan-Depleted Bovine Articular Cartilage Using High Frequency Ultrasound

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Running Title: **Ultrasonic Assessment of Altered Cartilage Swelling**

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Abstract

Swelling behavior is an electrochemical mechanical property of articular cartilage. It plays an important role in weight bearing and joint lubrication. In this study, the altered transient and inhomogeneous swelling behavior of the degenerated articular cartilage was observed and quantified in-situ using ultrasound. Three groups of bovine patellar articular cartilage samples (n = 10 x 3) were obtained and digested by trypsin for 10, 20, and 30 minutes respectively to mimick different levels of degeneration. The osmotic free shrinkage and swelling behavior induced by changing the concentration of the bathing saline solution from 0.15 M to 2 M and then back to 0.15 M, were characterized using high frequency ultrasound (central frequency = 35 MHz) before and after digestion. It was found that the degenerated cartilage specimens showed a weaker shrinkage-swelling behavior compared with the normal cartilage samples. However, no significant differences in the peak shrinkage or swelling strains were observed between different groups. The absolute values of the peak shrinkage strain significantly ($p < 0.05$) decreased by 45.4%, 42.1% and 50.6% respectively after the trypsin digestion for 10, 20 and 30 minutes, but such significance was not demonstrated for the peak swelling strains. Due to the potential alterations in the collagen-PG matrix during trypsin digestion, the correlation between the swelling strain and the shrinkage strain of the degenerated samples changed slightly in comparison with the normal samples. The proposed ultrasound method has been successfully used to measure the transient and inhomogeneous swelling behavior of the degenerated articular cartilage and has the potential for the characterization of

osteoarthritis.

Keywords: Ultrasound, Articular cartilage, Osmotic shrinkage-swelling, Trypsin digestion, Osteoarthritis.

Introduction

Articular cartilage covers the articulating bony ends in diarthrodial joints to provide a good distribution of the load on the bone and good lubrication for joint movement. Generally, the normal functions of articular cartilage can last for decades. However, factors such as aging, repetitive stress over an extended period, excessive weight-bearing stress, a sudden knee injury, and genetic factors may give rise to the degeneration of articular cartilage. Untreated injuries will lead to further degeneration, and even result in osteoarthritis (OA), one of the most common joint diseases.

Previous studies have been reported that articular cartilage as a multi-phasic hydrated mixture is mainly composed of 5-10% proteoglycan (PG), 10-20% collagen and 60-80% water (Mow et al, 2005). PGs are bio-macromolecules negatively charged by SO_3^- and COO^- (Maroudas, 1976). These negatively charged groups on the PGs quantified as the fixed charge density (FCD) (Lai et al., 1991) attract the mobile cations to generate a substantial Donnan osmotic pressure, one of the causes of cartilage swelling (Lai et al., 1991). Therefore, the PG content of the articular cartilage is suggested to contribute to its electromechanochemical behavior (Mow et

al., 2005). However, the swelling pressure in the cartilage tissue balances with the collagen tension, so no swollen behavior could be detected under normal conditions (Eisenberg and Grodzinsky, 1985; Lai et al., 1991; Maroudas, 1976; Maroudas et al., 1986). This pre-swollen state of articular cartilage plays an essential role in the biomechanical functions (Setton et al., 1998; Wang et al., 2002). When external forces are exerted on articular cartilage, the swollen cartilage carries the forces like a cushion (Mankin et al., 2000). The interstitial water also plays an important role in controlling the mechanical behavior of articular cartilage, including permeability, strength, stiffness and Young's modulus (Lai et al., 1991). When the tissue is under a pressure, the interstitial water allows the deformation via shifting in and out of the tissue. Meanwhile, the depth-dependent shape and orientation of collagen fibres are also highly responsible for the mechanical properties of articular cartilage (Zhu et al., 1993).

Therefore, component-level changes of the articular cartilage including the increase of tissue hydration, the loss of PGs, and the damage of collagen fibrils have attracted many research efforts and are claimed to be the earliest signs of cartilage degeneration during OA (Armstrong and Mow, 1982; Martini, 2004; Sandy, 2003; Torzilli et al., 1990). The damage of collagen network and the resultant decreases of PGs tend to result in the alteration of cartilage swelling (Bank et al., 2000). Enzyme digestion (Saarakkala et al., 2004; Laasanen et al., 2002, 2003b; Toyras et al., 2002; Zheng et al., 2004), intra-articular injection of chemical reagents (Cherin et al., 1998; Hattori et al.,

2005a; Laurent et al., 2003; Saied et al., 1997), surgery (Batiste et al., 2004; Calvo et al., 2004; Laurent et al., 2006; Song et al., 2006) and joint immobilization (Narmoneva et al., 2002) have usually been employed to induce the pathological changes in articular cartilage. It has been well agreed that the macromolecular degradation catalyzed by proteoglytic enzymes happens at the early- and mid-stages of cartilage degeneration (Sandy, 2003), making it possible to experimentally observe the cartilage degeneration process at an early stage.

Osmotic loading technique by varying the concentration of the bathing saline solution provides a simple and useful method to investigate mechanical-combined swelling (Eisenberg and Grodzinsky, 1985, 1987; Grodzinsky et al., 1981; Guilak et al., 1994; Mow and Schoonbeck, 1984; Myers et al., 1984; Wang et al., 2002) and free swelling behaviors of articular cartilage (Narmoneva et al., 1999, 2001; Setton et al., 1998; Wang and Zheng, 2006). In the early studies, the water gains of different zones of articular cartilage were weighted to indirectly quantify the swelling behavior (Maroudas, 1976; Maroudas et al., 1986; Mow and Schoonbeck, 1984). Integrated with tension and compression, the isometric swelling behaviors of cartilage strips with a fixed length were examined by changing the bath ionic concentration (Eisenberg and Grodzinsky, 1985, 1987; Grodzinsky et al., 1981; Guilak et al., 1994; Mow and Schoonbeck, 1984). Recently, the equilibrium swelling strain of the full-thickness cartilage was optically measured (Narmoneva et al., 1999) and the transient swelling and shrinkage strains were obtained using ultrasound (Wang and Zheng, 2006). When

articular cartilage was degenerated, the OA cartilage showed a more obviously inhomogeneous change in swelling strain (Maroudas, 1976; Narmoneva et al., 2001). However, many previous studies only investigated the equilibrated swelling of articular cartilage, it is still not clear how the transient swelling behavior is altered after the degeneration. Clinically, the detection of transient phenomena of cartilage within a short time will be more significant than the detection of equilibrated one, which normally requires a long time to obtain a result.

Our previous study has demonstrated that it is feasible to use high-frequency ultrasound to monitor the transient behavior of articular cartilage during the free swelling or shrinkage process induced by the concentration change of the bathing saline solution as well as the progressive enzyme digestion (Zheng et al., 2004). In our recent study, the quantitative ultrasonic characteristics of the transient changes of normal bovine patellar cartilage under the osmotic loading were investigated (Wang and Zheng, 2006). In comparison with the water-gain weighting and optical imaging methods, ultrasound can provide the transient and depth-dependent swelling information of the intact cartilage tissue *in situ*. In this study, the ultrasound method was used to probe and quantify the altered transient inhomogeneous swelling behavior of the trypsin-treated articular cartilage in comparison with the normal tissues.

Specimen and Method

Specimen Preparation

Based on Noyes grading scale (Noyes and Stabler, 1989), fresh mature (1.5 ~ 2 years) normal bovine patellae (n = 30) were selected. Their cartilage surfaces were intact and smooth with no visible defects and irregularities. One cartilage-bone plug was prepared from the medial upper quadrant of each patella by using a metal punch with a diameter of 6.35 mm (Wang and Zheng, 2006). The specimens were randomly and equally divided into three groups, Group 10min, Group 20min, and Group 30min, which would be treated by trypsin for 10, 20, and 30 minutes, respectively. Specimens were wrapped in wet gauze soaked with physiological saline, and stored at -20ºC until testing. All the samples were tested within one month. Before testing, the cylindrical plug was cut into two parts. The 2/3 portion was used as the testing sample and the 1/3 portion reserved as the control for the histological assessment only.

Ultrasound System

The manually controlled 3D ultrasound swelling measurement system (USMS) introduced in our previous study (Fig. 1, Wang and Zheng, 2006) was employed to monitor the transient shrinkage and swelling behavior of articular cartilage in a non-contact and non-destructive way. This system mainly included a focused ultrasound transducer with a central frequency of 35 MHz, a focal length of 12.7 mm, a focal zone diameter of 0.1 mm, and a focal zone depth of 0.95 mm (Model PI50-2, Panametrics, Waltham, MA, USA), an ultrasound pulser/receiver (Model 5601A, Panametrics, Waltham, MA, USA), and a computer with an 8-bit A/D converter (Model CompuScope 8500PCI, Gage, Canada) and a custom-designed program

developed in our previous study (Zheng et al., 2001, 2004) for the ultrasound measurement of motion and elasticity (UMME). The ultrasound pulser/receiver was used to drive the ultrasound transducer and receive the ultrasound echoes reflected by the tissue. The received ultrasound signals were digitized by the A/D card with a sampling rate of 500 MHz. The corresponding resolution for the time-of-flight measurement for ultrasound echoes was 2 ns. After the peak of the cross-correlation was identified, the corresponding signal was locally upsampled by 10 times to achieve a time resolution of 0.2 ns, Assuming an average ultrasound speed in articular cartilage of 1675 m/s (Patil et al., 2004), the corresponding displacement resolution in the tissue could approach to approximately 0.17 μm. The ultrasound signals were displayed on the monitor in real time and automatically saved into the hard disk for offline data analysis. A high correlation (ICC > 0.98) of the maximum strains obtained in our previous study (Wang and Zheng, 2006) demonstrates a good repeatability of our ultrasonic measurement.

During the test, the 2/3 portion of the cartilage-bone plug was fixed on the bottom of the container, surrounded by rubber gel (Blu-Tack, Australia), and then submerged in the saline solution (Fig. 1). The outer ring of the surface of the cartilage disc with a width of approximately 0.6 mm was gently covered by the rubber gel. Therefore, the diffusion of ions and water and the penetration of trypsin enzyme were not allowed from the sides of the specimen. Adjusting the three micrometers on the translating stage (Model R301MMX/2201MMXY, Ball Slide Positioning Stages, Deltron

Precision Inc.), the focused ultrasound beam was aligned into the central portion of the cartilage specimen with the maximum echo amplitude (Wang and Zheng, 2006).

Experiment Protocol

All the experiments were carried out at room temperature (21±1ºC). The 2/3 portion specimens were thawed for three hours in physiological saline before tested using the manually controlled 3D USMS. The experiment protocol was as follows.

A. Normal shrinkage-swelling test

As described in our previous study (Wang and Zheng, 2006), the free shrinkage and swelling behavior was induced by changing the concentration of the bathing solution. After the specimen was mounted and equilibrated, the physiological saline solution was rapidly removed using an injection syringe and replaced with the 2 M saline within 30 seconds. The cartilage sample was given one hour to reach a new equilibrium, during which the dynamic contraction of the cartilage layer at different depths caused by the Donnan osmotic loading was observed in the ultrasound signals. After that, the bathing saline was quickly changed back to 0.15 M saline solution within 30 seconds, and the corresponding one-hour swelling procedure was also monitored.

B. Enzyme digestion

After normal shrinkage-swelling test, the 0.15 M NaCl solution was rapidly replaced

with 0.25% trypsin solution. The specimens of Group 10min, Group 20min and Group 30min were immerged in the enzyme solution for 10 minutes, 20 minutes and 30 minutes, respectively. After the digestion, the enzyme solution was rapidly removed out of the container and the physiological saline solution was refilled in. The residual enzyme digestion lasted for three hours and was followed by the post-digestion shrinkage-swelling test.

C. Post-digestion shrinkage-swelling test

While the PG-depleted specimen was remained in the container, the bathing solution was quickly replaced with 2 M NaCl solution and the dynamic contraction process was observed by ultrasound as described above. Correspondingly, the swelling test was also performed using the abovementioned protocol by changing the saline from 2 M to 0.15 M. Then the tested specimen was wrapped in wet gauze soaked with physiological saline and store at -20ºC until the histological evaluation.

D. Histological evaluation

After the whole ultrasonic monitoring was completed, both the control (1/3 portion) and the trypsin-digested (2/3 portion) specimens were thawed for histological evaluation. The specimens were first preserved and fixed in a 10% buffered formalin (pH 7.0) for more than 4 hours at room temperature. Then, they were decalcified in a 10% ethylenediaminetetracetic acid (EDTA) solution using ultrasound-assisted decalcificater (Guo et al., 2005) till the attached bone tissue could be easily cut using

a scalpel. The tissue sections, 4μm in thickness, were prepared using rotatory microtome (Leica RM-2135, UK), stained with safranin O (SiGMA, CAT NO. F7258, USA) and contra-stained with fast green (SiGMA, CAT NO. S-2255, USA) (Leung et al., 1999). Sections were examined by a light microscopy imaging system including microscope (ECLIPSE 80i, Nikon, Japan) and digital camera (DXM 1200C, Nikon, Japan). The PG content corresponded to the area fraction of the safranin O stained zone in the histological image (Leung et al., 1999).

Parametric Measurement

Five quantitative parameters were extracted from the shrinkage-swelling measurements. They were the transient strain and the maximum (or peak) strain of the full-thickness cartilage layer, the time to reach the peak value named as duration, the equilibrium swelling strains at different depths, and the percentage change of ultrasound speed in the cartilage tissue. In this study, the time shift (T) of the ultrasound echoes was measured using cross-correlation algorithm (Ophir et al., 1991; Zheng et al., 2001). The displacement (d) of the cartilage surface equals to the product of the sound speed c_s in the saline (either 0.15 M or 2 M) and the time shift valve (Eq. 1).

$$d = c_s * T / 2 \qquad (1)$$

In consideration of the period (approximately 30 seconds) of changing the saline solution, the compensation to the displacement measurement was performed (Wang and Zheng, 2006). Then, the strain (ε) of the full-thickness cartilage layer can be

calculated using Eq. 2.

$$\varepsilon = \frac{d}{h} \qquad (2)$$

where h is the thickness of the cartilage layer and given by $h = c_{ac}\frac{T_{ac}}{2}$, where c_{ac} is the sound speed in the cartilage tissue and T_{ac} denotes the corresponding time-of-flight of the ultrasound echoes throughout the cartilage matrix during shrinkage or swelling phase.

At equilibrium, the strain of each sub-layer equals to the displacement of the sub-layer divided by its thickness. Considering the depth-dependence of the sound speed in articular cartilage, the equilibrium strain (ε_i) of the i^{th} arbitrary layer (from depth x_i to depth x_{i+1}) after compensation can be expressed by Eq. 3 (Wang et al., 2007).

$$\varepsilon_i = \frac{T'_{x_{i+1}} - T'_{x_i}}{t_{x_{i+1}} - t_{x_i}} \qquad (i = 1, 2, \ldots N) \qquad (3)$$

where N is the number of the sub-layers. T'_{x_i} and $T'_{x_{i+1}}$ are the compensated time shifts of the echoes from the tissue at depths x_i and x_{i+1}, respectively. t_{x_i} and $t_{x_{i+1}}$ are the original flight times of ultrasound at depths x_i and x_{i+1}, respectively. Using the sound speed in the saline measured in this study and the average sound speed in articular cartilage (1675±51 m/s and 1781±48 m/s in 0.15 M and 2 M saline, respectively) (Patil et al., 2004), the changed sound speed in articular cartilage could be calculated from Eq. (4) (Wang and Zheng, 2006).

$$c'_{ac} = \frac{d + c_{ac} * T_i}{T_{i+1}} \qquad (4)$$

where c_{ac} and c'_{ac} are the sound speeds in articular cartilage at the moment of t_i and t_{i+1}, respectively; T_i and T_{i+1} are the flight time of ultrasound in the cartilage matrix at t_i and t_{i+1}, respectively. d is the displacement of the cartilage surface occurred between t_i and t_{i+1}.

Data Analysis

Due to the relatively small number of samples in this study, the non-parametric Wilcoxon's signed rank test was used for the statistical comparisons of paired samples. Kruskal-Wallis test was used to test the statistical difference between groups with different trypsin digestion periods. Spearman's correlation was used to test the relationship between peak shrinkage strain and peak swelling strain. The statistical analyses were conducted using SPSS software (V11.5, SPSS Inc., Chicago, USA).

Results

Fig. 2 shows the M-mode images of the ultrasound signals collected during swelling and shrinkage processes before trypsin digestion. The deformations of the cartilage at different depths, especially the superficial layer (indicated by white arrows in Fig. 2) were dynamically indicated in the ultrasonic images. The digestion process was clearly shown by the echoes generated at the interface between the digested and undigested tissues (Fig. 3). These echoes formed an inclined streak of the digestion front and can be observed. After digested with trypsin, the degenerated cartilage specimens showed weaker shrinkage and swelling behaviors (Fig. 3c,d) in comparison

with the normal specimens (Fig. 2b,c). The average transient shrinkage or swelling strains of the normal cartilage over the 10 specimens were compared with those of the degenerated samples in Fig. 4.

The overshoot relaxation phenomena during the shrinkage and swelling phases were observed in this study for both the normal and degenerated cartilage tissues. For the degenerated cartilage, the mean overshoot amplitude of the shrinkage behavior changed with a greater decrease than that of the swelling behavior (Fig. 4). The absolute values of the peak shrinkage strain significantly ($p < 0.05$) decreased by 45.4%, 42.1% and 50.6% respectively after the trypsin digestion for 10, 20 and 30 minutes (Fig. 5a), but no significant difference between different groups was found ($p > 0.05$). The peak swelling strains for the three groups did not significantly ($p > 0.05$) change before and after the digestions and similarly no significant difference between different groups was found ($p > 0.05$, Fig. 5b). Therefore, in view of all the samples together, a significantly larger absolute value of the overall mean peak strain of shrinkage (0.009 ± 0.003) was obtained compared with that of the swelling process (0.004 ± 0.002) for the normal specimens ($p < 0.001$). After trypsin digestions, this absolute value for the overall shrinkage strain significantly reduced to 0.005 ± 0.002 ($p < 0.05$) while that for the overall mean peak swelling strain insignificantly reduced to 0.003 ± 0.001 ($p > 0.05$).

The statistical results of shrinkage duration and swelling duration between different

groups showed no significant change ($p > 0.05$, Fig. 6). For Group 20min and Group 30min, the duration did not change significantly ($p > 0.05$). However, the duration of the 10-minute digested samples significantly decreased in comparison with the corresponding normal samples ($p < 0.05$). Meanwhile, large standard deviations in the duration measurement for both the normal and degenerated cartilage tissues were observed.

Along the depth direction, the swelling strains of different layers (surface (approximately 15% of the total thickness), middle (approximately 55%), and deep layer (approximately 30%), Mow et al., 2005; Wang et al., 2007) were measured at equilibrium. It was found that compared with the normal tissues of Group 30min, the mean swelling strain for the degenerated tissues increased at the superficial layer, decreased at the middle layer significantly ($p < 0.05$), and changed little at the deep layer (Fig. 7a). However, for the other two groups digested with shorter time, the strains of the three layers changed insignificantly ($p > 0.05$, Fig. 7b,c).

After the depletion of the PG content, the percentage change of the sound speed in articular cartilage during both the shrinkage and swelling processes remained to be exponentially dependent on the measurement time (Fig. 8), expressed as $y = A + Be^{Ct}$. The overall speed change (5.1 ± 0.8 % and -4.9 ± 1.2 % for the shrinkage and swelling processes, respectively) for the degenerated cartilage was similar to the values (5.2 ± 1.5 % and -5.1 ± 1.1 %) for normal samples. There was no

significant difference among the three groups ($p > 0.05$).

Fig. 9 shows the correlation between the peak swelling strain and the peak shrinkage strain of the normal cartilage ($R = -0.45$, $p < 0.05$) and a similar but insignificant correlation ($R = -0.315$, $p = 0.09$) was obtained for the trypsin-digested samples.

Discussion

This study systematically investigated the osmosis-induced shrinkage-swelling behavior of the degenerated cartilages in comparison with the normal tissues using ultrasound. It was found that the absolute value of the peak shrinkage strain of the full-thickness cartilage layer decreased (45.4%, 42.1% and 50.6% for the three groups, respectively) significantly after the PG content was digested by trypsin. The possible reasons for a relatively less decrease for the 20-minute treated samples in comparison with the other two groups might be as follows. (1) The interval of the digestion time (10 minutes) was not enough to induce significant difference between groups. It may be because of the 3-hour residual digestion in physiological saline performed in this study, which appeared to weaken the difference in pure trypsin digestion between different groups. Its effect will be investigated in our future study in details. (2) The individual differences among specimens include cartilage thickness and PG concentration. (3) The small number of specimens was used in this study. It is suggested that a larger number of samples should be involved in the experiment to reduce the effect of the individual differences between specimens and that a longer

digestion time should be designed to generate the degenerated specimens with significant changes.

The result that the peak swelling strain only decreased insignificantly is similar to the study of Narmoneva et al. (2001). They reported that the swelling strain of the mild degenerated cartilage changed little in comparison with the normal tissue. However, other previous studies have claimed that the increase in cartilage volumetric swelling (or water gain) would be regarded as one of the early signs of OA (Maroudas et al., 1986; Torzilli et al., 1990). Different digestion methods or severities of the collagen-PG matrix might be responsible for this discrepancy. It was reported that damage and loss of collagen fibres might result in stronger swelling behavior (Narmoneva et al., 2001). Mankin et al. (2000) also suggested that the loss of PGs and the limitation of the unchanged collagen network might be the determinative factors in the decrease of swelling pressure and the loss or gain of water. According to earlier study, no qualitative changes in the collagen network of the trypsin-digested cartilage were found in polarized light microscopy images, although statistically significant decrease of ultrasound reflection from the cartilage surface indicated the effect of trypsin digestion on the collagen network (Nieminen et al., 2002). It has been known that trypsin enzyme causes minor degeneration of collagen network (Harris et al., 1972). However, the quantitative effect of collagen damage on the shrinkage and swelling behavior has not been reported. In this study, the ultrasound M-mode image showed that besides the inclined digestion trace of the additional echoes, the echoes from the cartilage matrix shifted slightly, indicating that the progressive digestion did

not obviously influence the ultrasound scattering signals from the tissue. This was consistent with the result of sound speed measurement. Some previous studies also suggested that trypsin decomposed PG macromolecules but only caused a minor change in the condition of the whole matrix, such as the impedance or density of the cartilage matrix (Gu et al., 1999) and cartilage structure and composition (Lyyra et al., 1999). Therefore, the damage of collagen network induced by trypsin has not been quantitatively determined. Using collagenase enzyme tends to be an alternative method to study the swelling behavior of the collagen-damaged articular cartilage.

Previous studies usually used the condition of articular cartilage in 2 M saline solution as the reference configuration and did not regard it as a testing phase (Eisenberg and Grodzinsky, 1985; Lai et al., 1991; Narmoneva et al., 1999). In the current study, not only the swelling behavior but also the shrinkage (also known as de-swelling) behavior was investigated. The result of the correlation between the peak swelling and shrinkage strains demonstrated that the cartilage tended to swell with larger amplitude if it had undergone a stronger shrinkage. Meanwhile, it was found for normal and digested cartilage that there was a significant difference between the transient responses during the shrinkage and swelling processes in the strain amplitude. This difference might be caused by the special ‘overshoot-relaxation’ phenomenon which had been discussed in our previous study (Wang and Zheng, 2006) and was also observed in the current work and became insignificant after PGs were digested. The cartilage surface layer with a collagen network may significantly limit cartilage

swelling (Setton et al., 1998), but it may also have slight limitation on shrinkage behavior due to the osmotic pressure generated by the hypertonic bathing solution and applied on the cartilage in an opposite direction compared with the swelling process. In addition, another two factors should be taken into account in this study, the equilibrating time after the bathing saline solution is changed and the time of solution change. Many previous studies on free swelling of articular cartilage allowed specimens with different shapes and sizes to reach equilibrium within approximately one hour (Eisenberg and Grodzinsky, 1985, 1987; Flahiff et al., 2004; Myers et al., 1984; Setton et al., 1998; Wang et al., 2002; Zheng et al., 2004). Some other studies gave the specimens a longer time (e.g. 4 hours) to approach equilibrium (Flahiff et al., 2002; Narmoneva et al., 1999). In this study, we noted that a majority of the specimens were close to an equilibrium state after 60 minutes. It appeared that cartilage specimens from different patella required different equilibrating time during the free swelling. Although efforts had been made to change the solution as quickly as possible, the time used to perform this might affect the swelling or shrinkage process. It is obvious in Fig. 9 that the normal articular cartilage has a larger peak shrinkage strain than the digested one for the same peak swelling strain, particularly for the specimens with small peak swelling strains. The potential reason for the phenomenon might be the damage of the collagen network during the trypsin digestion, which has been report to have some degrees of effects to the collagen (Harris et al., 1972). Because of the damage of collagen, the matrix is not able to shrink as strong as that in the normal cartilage under the high saline concentration. Nevertheless, further studies

are required for a better understanding of the relationship between these two phases.

In addition, it was found that the transient shrinkage strain of the degenerated cartilage become positive when the surface returns to the original position after approaching the peak value (Fig 4b.). The possible reasons may be that the PG depletion caused a decrease of the stiffness of the collagen-PG solid matrix (Mow and Hung, 2001). The softer matrix might have excessive response to the relaxation from the overshoot and gradually approach the original position at equilibrium. The relatively larger overshoot amplitude of the shrinkage behavior might explain why this kind of phenomenon was only found during the shrinkage phase.

The current result of the depth-dependence of the swelling-induced strains for the degenerated samples was consistent with the result of a previous study on the swelling strain of the damaged cartilage (Narmoneva et al., 2001). The non-uniform distribution of the equilibrium swelling strain at different depths of the full-thickness cartilage might be explained by the constraint of collagen network in the surface layer, the distributions of PGs, collagen fibres and water, and the support of the subchondral bone layer. It has been well known that the middle layer contained more PGs than the surface layer (Mow et al., 2005; Narmoneva et al., 2001). Therefore, with the loss of PGs, the swelling strain at the middle zone significantly reduced while the swelling strain at the surface zone increased. The strain at the deep zone changed relatively little probably because the deep zone was not digested by trypsin, which was

confirmed by both the histological and ultrasound measurements.

Conclusion

The "overshoot-relaxation" transient shrinkage-swelling behavior induced by changing the concentration of the bathing solution for both normal and degenerated cartilage tissues was observed using high-frequency ultrasound in this study. It was found that the trace of the digestion front could be clearly tracked, and the peak shrinkage strain decreased significantly while the peak swelling strain only had an insignificant decrease after the cartilage tissues were digested by trypsin for 10 to 30 minutes. No significant difference in the percentage change of the ultrasound speed during shrinkage and swelling phases was observed after the tissues were trypsin-treated. The results demonstrated that PG depletion affected the shrinkage and swelling behaviors of articular cartilage remarkably but had little effect on the ultrasound speed. The proposed ultrasound method has been successfully used to measure the transient swelling behavior of the OA-like articular cartilage. However, the spontaneous OA is a process related to a multi-composition change of articular cartilage too complex for the experimentally enzyme-treated articular cartilage to stand for. Thus further studies are required to investigate the swelling behavior of the spontaneous OA articular cartilage using our ultrasound method.

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Fig. 1 Schematic of ultrasound swelling measurement system (USMS) monitoring the transient changes of articular cartilage induced by osmotic loading. A fine copper wire was attached under the transducer and approximately 1.5 mm from the surface of the cartilage specimen to generate a calibration echo for the compensation of the effect of temperature fluctuation on the measurement results (Wang and Zheng, 2006).

Fig. 2 The M-mode ultrasound images collected from a consistent site of one specimen during different monitoring processes: (a) Equilibrium in 0.15 M saline; (b) Shrinkage process after 0.15 M saline was replaced with 2M saline; (c) Swelling process after 2 M saline was replaced with 0.15 M saline; (d) Histological image of normal cartilage section stained with safranin O and fast green. The white arrows indicate the deformations of articular cartilage during the beginning period of the shrinkage and swelling phase.

Fig. 3 The M-mode ultrasound images collected from the same site as in Fig. 2 during trypsin digestion and other post-digestion monitoring processes: (a) Trypsin digestion after the 0.15 M saline was replaced with 0.25% trypsin solution; (b) Residual digestion after trypsin solution was replaced with 0.15 M saline; (c) Shrinkage process; (d) Swelling process; and (e) Histological image of the degenerated cartilage section. The area stained with red color (dark in grey image) indicates the PG content reduced in comparison with Fig. 2d. Black arrows show the digestion trace in (b-d) and the interface of the digested and undigested tissues in (e). The white arrows indicate the

deformations of articular cartilage during the beginning period of the shrinkage and swelling phase, which are with smaller amplitude than those in Fig. 2.

Fig. 4 The comparisons of the transient shrinkage (a) and swelling (b) strains between the normal and degenerated cartilage tissues. The light grey and black solid curves represent the mean strains measured before and after trypsin digestion, respectively.

Fig. 5 The comparisons of the peak shrinkage (a) and swelling (b) strains between the normal and degenerated cartilage tissues. The error bars represent standard deviation ($n = 10$). * Significant difference ($p < 0.05$ by Wilcoxon's signed rank test) between the normal and degenerated samples.

Fig. 6 The comparisons of the shrinkage (a) and swelling (b) duration between the normal and degenerated cartilage tissues. The error bars represent standard deviation ($n = 10$).

Fig. 7 The mean equilibrium swelling strains at the deep, middle and surface layers of the degenerated cartilage samples in comparison with normal samples. (a) Group 10min, (b) Group 20min and (c) Group 30min

Fig. 8 The comparison of the percentage change of ultrasound speed in articular cartilage between the normal and degenerated samples. The error bar represents

standard deviation (n = 10).

Fig. 9 Correlations between the peak shrinkage strain and the peak swelling strain for the normal and degenerated specimens.

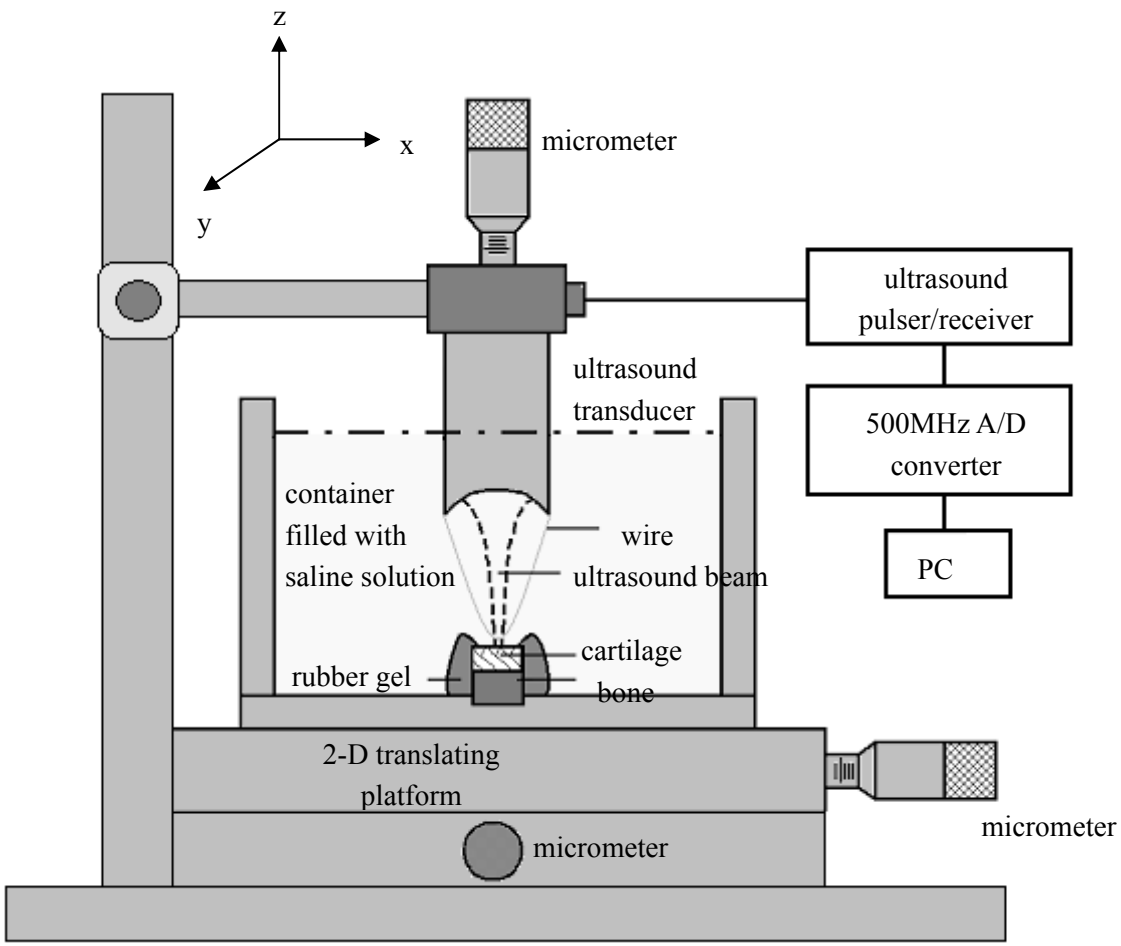

z
x
y
micrometer
ultrasound
pulser/receiver
ultrasound
transducer
500MHz A/D
converter
container
filled with
saline solution
wire
ultrasound beam
PC
cartilage
rubber gel
bone
2-D translating
platform
micrometer
micrometer

Fig. 1

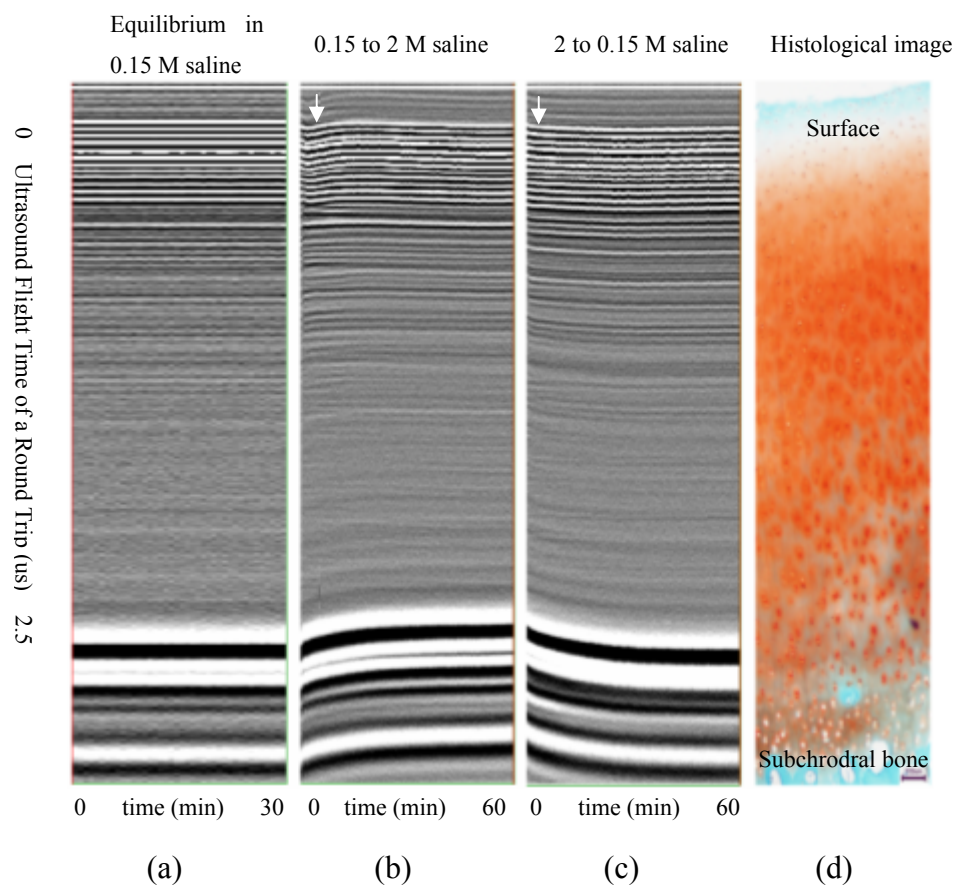

Equilibrium in 0.15 M saline
0.15 to 2 M saline
2 to 0.15 M saline
Histological image
Surface
Subchrodral bone
0 Ultrasound Flight Time of a Round Trip (us) 2.5
0 time (min) 30
0 time (min) 60
0 time (min) 60
(a)
(b)
(c)
(d)

Fig. 2

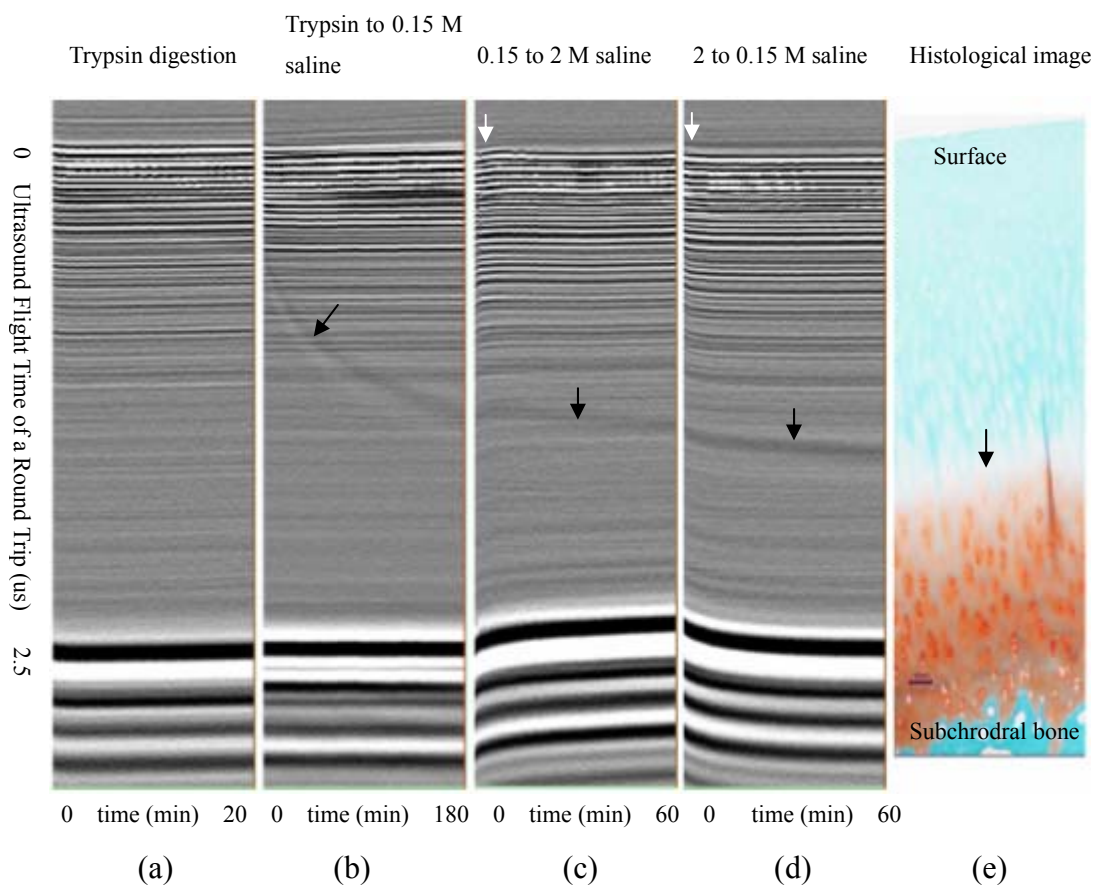
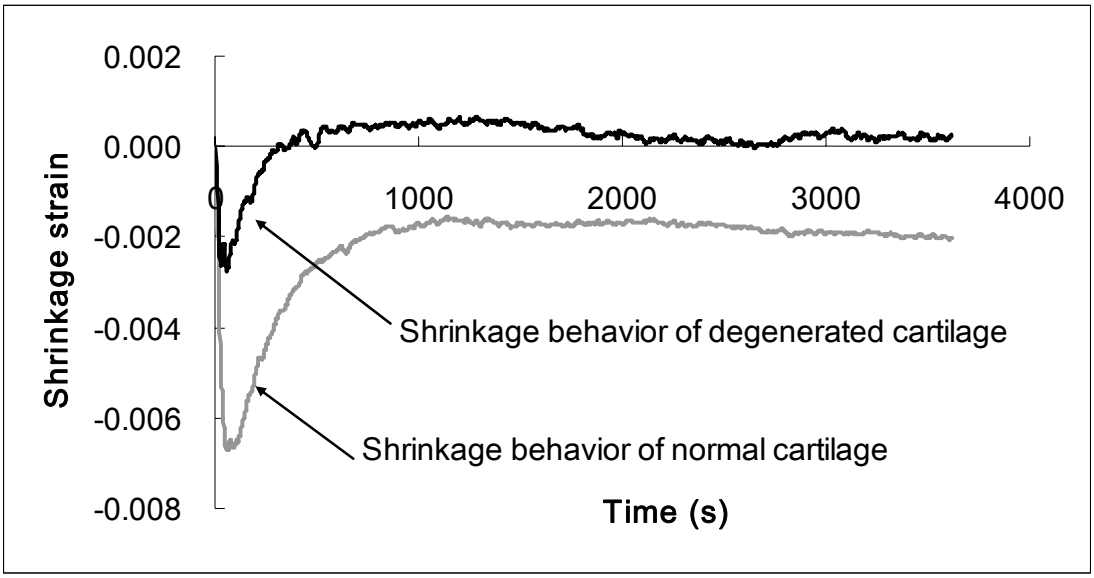
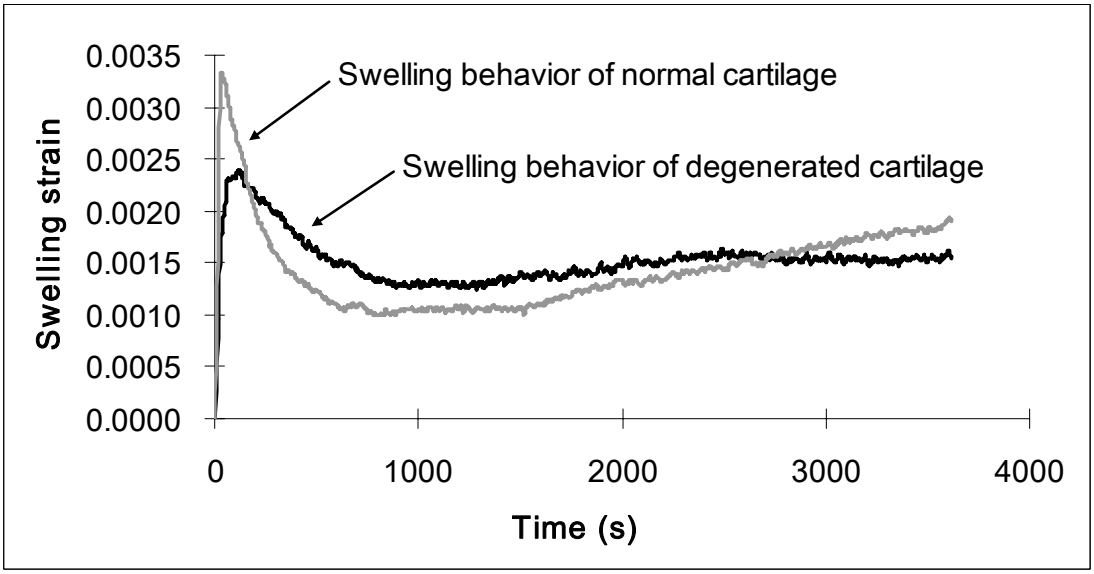

Trypsin digestion
Trypsin to 0.15 M saline
0.15 to 2 M saline
2 to 0.15 M saline
Histological image
0
Ultrasound Flight Time of a Round Trip (us)
2.5
Surface
Subchrodral bone
0 time (min) 20
0 time (min) 180
0 time (min) 60
0 time (min) 60
(a)
(b)
(c)
(d)
(e)

Fig. 3

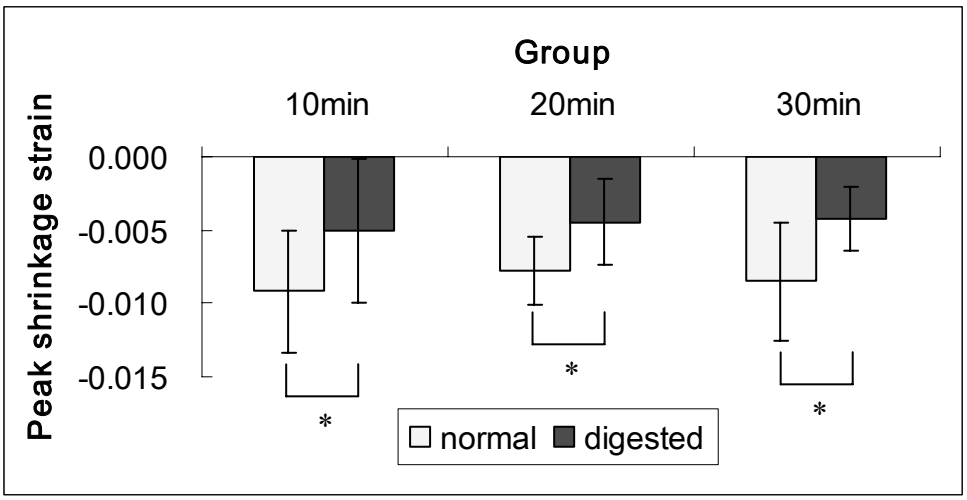

0.002
0.000
-0.002
-0.004
-0.006
-0.008
Shrinkage strain
0
1000
2000
3000
4000
Shrinkage behavior of degenerated cartilage
Shrinkage behavior of normal cartilage
Time (s)

(a)

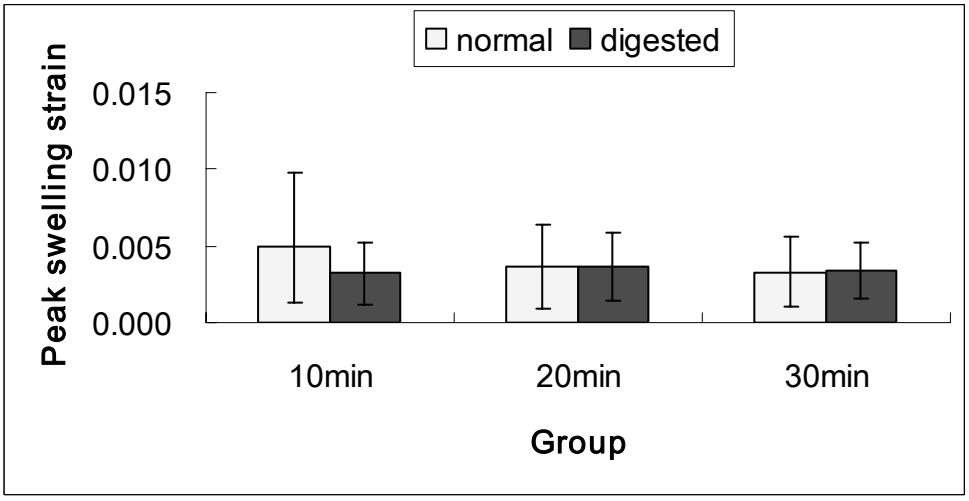

0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Swelling strain
Swelling behavior of normal cartilage
Swelling behavior of degenerated cartilage
0
1000
2000
3000
4000
Time (s)

(b)

Fig. 4

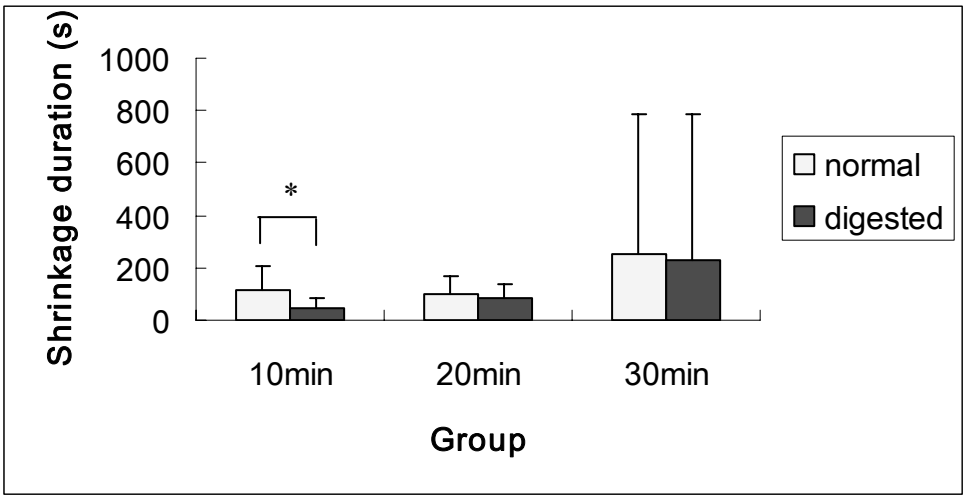

Group
10min
20min
30min
Peak shrinkage strain
0.000
-0.005
-0.010
-0.015
*
*
*
normal
digested

(a)

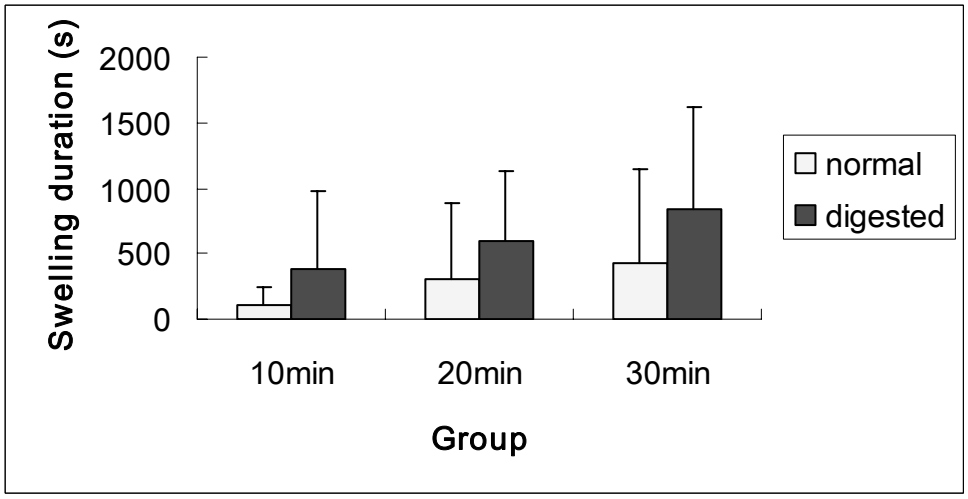

normal
digested
Peak swelling strain
0.015
0.010
0.005
0.000
10min
20min
30min
Group

(b)

Fig. 5

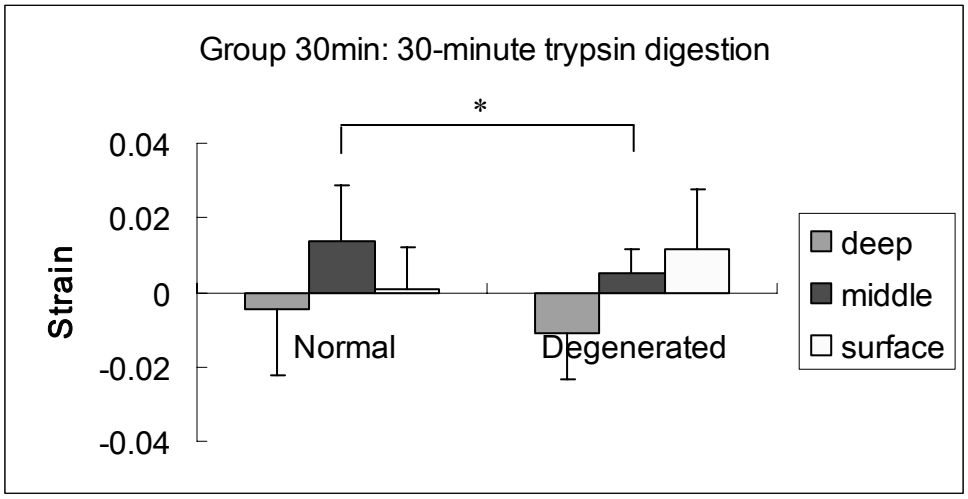

Shrinkage duration (s)
1000
800
600
400
200
0
*
10min
20min
30min
Group
normal
digested

(a)

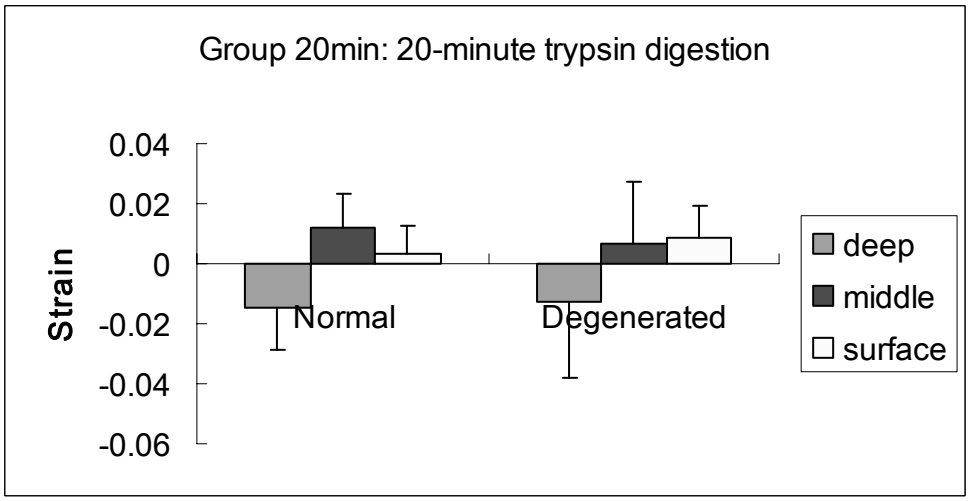

Swelling duration (s)
2000
1500
1000
500
0
10min
20min
30min
Group
normal
digested

(b)

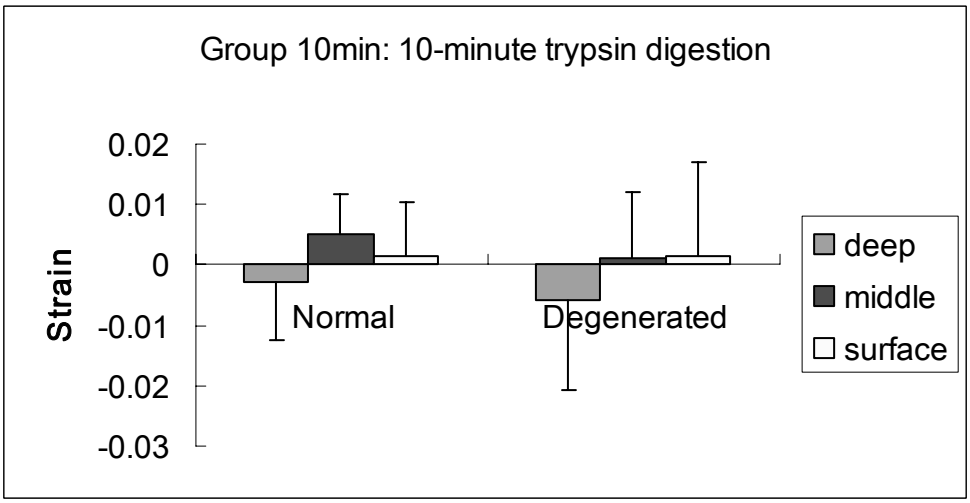
Fig. 6


Group 30min: 30-minute trypsin digestion
*
0.04
0.02
0
-0.02
-0.04
Strain
Normal
Degenerated
deep
middle
surface

(a)


Group 20min: 20-minute trypsin digestion
0.04
0.02
0
-0.02
-0.04
-0.06
Strain
Normal
Degenerated
deep
middle
surface

(b)


Group 10min: 10-minute trypsin digestion
0.02
0.01
0
-0.01
-0.02
-0.03
Strain
Normal
Degenerated
deep
middle
surface

(c)

Fig. 7

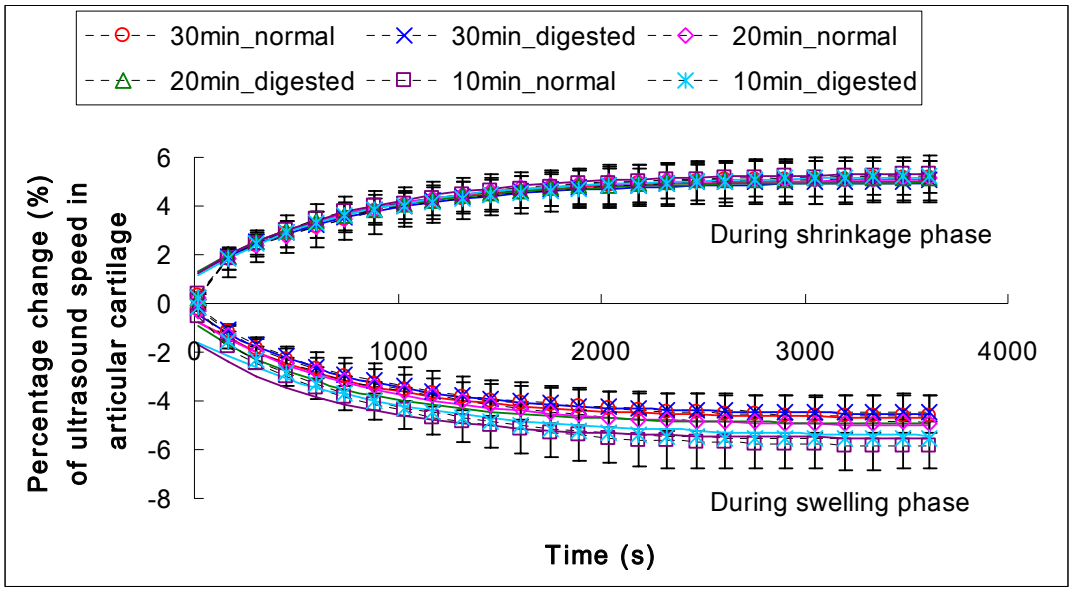

30min_normal
30min_digested
20min_normal
20min_digested
10min_normal
10min_digested
Percentage change (%) of ultrasound speed in articular cartilage
6
4
2
0
-2
-4
-6
-8
0
1000
2000
3000
4000
During shrinkage phase
During swelling phase
Time (s)

Fig. 8

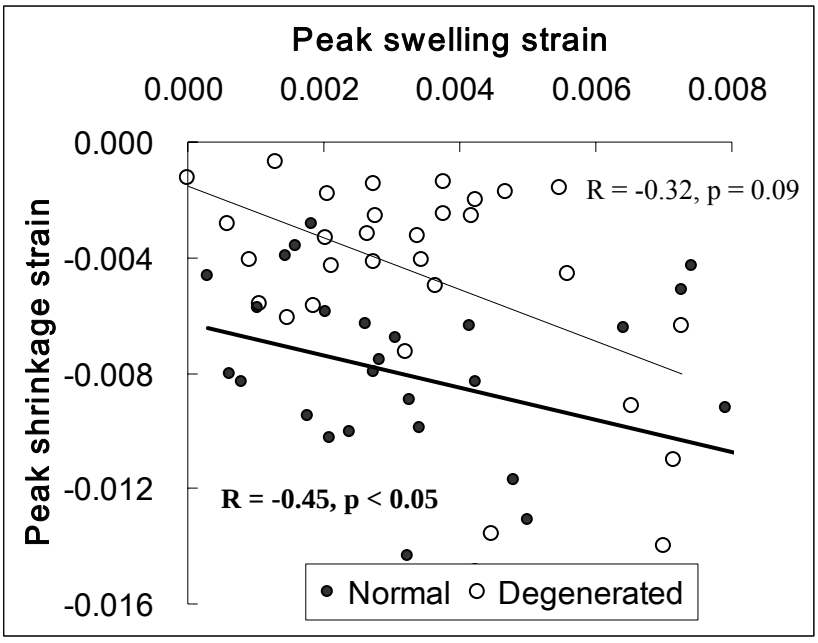

Peak swelling strain
0.000
0.002
0.004
0.006
0.008
Peak shrinkage strain
0.000
-0.004
-0.008
-0.012
-0.016
R = -0.32, p = 0.09
R = -0.45, p < 0.05
Normal
Degenerated

Fig. 9