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Relationship between triphasic mechanical properties of articular cartilage and osteoarthritic grade

NIU HaiJun[1], LIU ChengRui[1], LI Ang[1], WANG Qing[2], WANG YueXiang[3], LI DeYu[1] & FAN YuBo[1*]

[1]*Key Laboratory of Ministry of Education for Biomechanics and Mechanobiology, School of Biological Science and Medical Engineering, Beihang University, Beijing 100191, China;*
[2]*School of Biomedical Engineering, Southern Medical University, Guangzhou 510515, China;*
[3]*Department of Ultrasound, People Liberation Army General Hospital, Beijing 100853, China*

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The purpose of this study was to explore the triphasic mechanical properties of osteoarthritic cartilage with different pathological grades. First, samples of cartilage from rabbits with different stages of osteoarthritis (OA) were graded. Following this, the cartilage was strained by a swelling experiment, and changes were measured using a high-frequency ultrasound system. The result, together with fixed charge density and water volume fraction of cartilage samples, was used to estimate the uniaxial modulus of the cartilage tissue, based on a triphasic model. For the control cartilage samples, the uniaxial elastic modulus on the cartilage surface was lower than those in the middle and deep layers. With an increase in the OA grade, the uniaxial elastic modulus of the surface, middle and deep layers decreased. A significant difference was found in the surface elastic modulus of different OA grades (P<0.01), while no significant differences were identified for OA cartilages of Grades 1 and 2 in the middle and deep layers (P<0.01). Compared with Grades 1 and 2, there was a significant reduction in the elastic modulus in the middle and deep layers of Grade 3 OA cartilage (P<0.05). Overall, this study may provide a new quantitative method to evaluate the severity of OA using the mechanical properties of cartilage tissue.

osteoarthritis grade, triphasic theory, ultrasound, uniaxial modulus

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Osteoarthritis (OA) is a chronic joint disease that occurs with the progressive degeneration of articular cartilage (AC), followed by secondary tissue hyperplasia. Clinical symptoms of OA include joint pain, loss of function and joint deformity. Early diagnosis of OA is essential for the timely treatment and prevention of advanced complications [1,2].

AC consists of a relatively small number of chondrocytes (5% of the total wet weight) and a large amount of extracellular matrix (ECM) (95%) that is rich in water, collagen fibers, proteoglycans (PG), and other minor components [2]. The proportions of these components vary within the different layers of AC, and together with the structure, determine the biomechanical characteristics of the tissue [2,3]. In a normal physiological state, an equilibrium is formed through the interaction between swelling pressure, which is produced by PGs combined with water in tissue interstitial spaces, and the holding force of the collagen network. This equilibrium is necessary for maintaining the steady state of cartilage mechanical properties [4]. However, under pathological states, such as OA, this equilibrium is disrupted because of changes to the components and structure, which in turn alter the mechanical properties of AC [5,6]. Therefore,

*Corresponding author (email: yubofan@buaa.edu.cn)

fluctuations in the mechanical properties of the AC can act as an important sign of early OA.

Techniques, such as arthroscopy, X-ray computed tomography (CT) and magnetic resonance imaging (MRI) have been used in studies of OA. However, these technologies are not very effective for early OA diagnosis [7–10]. Many researchers have studied the mechanical properties of degraded cartilage, and attempted to use mechanical properties to evaluate the degree of cartilage degradation. Most have estimated the elastic modulus of the AC tissue, based on mono- or biphasic theories, combined with confined compression, unconfined compression and indentation measurements. The results showed that the tensile modulus and shear modulus decreased with the severity of OA [3,11–14]. However, these conventional mechanical testing methods need to make contact with the tissue and induce an external load to the cartilage, which makes them unsuitable for clinical diagnosis. In addition, these methods can only be used to calculate the bulk modulus of the tissue, and cannot describe the mechanical characteristics of the tissue in different layers, because strain data for cartilage internal tissue are lacking.

In recent years, ultrasound has received increased attention as a tool for the early detection of OA. Many have explored the relationship between the acoustic parameters (such as the speed of sound, attenuation, reflection coefficient and scattering coefficient) and the components of OA cartilage. These studies used ultrasound to observe the internal strain of the cartilage induced by the external load, and applied monophasic, biphasic or triphasic models to calculate the mechanical properties of cartilage [15–17]. Noticeably, the inhomogeneous triphasic model provides the most comprehensive description of the cartilage mechanical properties, with studies showing that uniaxial elastic modulus in different layers can be estimated with the triphasic model [11,18,19]. In previous work, we applied a triphasic model to predict the swelling trend of cartilage, and validated the effectiveness of this method [11,18].

Clinically, the severity of OA is usually graded according to the standards of International Osteoarthritis Institute (OARSI), based on histological analysis [17]. However, histological analysis is invasive and requires removal of a specimen from the cartilage tissue. Therefore, it is only suitable for the diagnosis of advanced OA, and not for the detection of early OA. In addition, it is semi-quantitative and highly dependent on the judgment of the clinician. In our previous studies, we showed that the severity of OA significantly correlates with the mechanical properties of AC [11,18]. Therefore, in this study, we estimated the unaxial elastic modulus of AC using high frequency ultrasound and the triphasic theory of AC. The aim was to explore the mechanical properties of OA cartilage in different OARSI pathological grades. The results may provide some useful information for ultrasonic evaluation of OA cartilage and for the quantitative diagnosis of early OA.

1 Materials and methods

1.1 Animal models

Eighteen normal adult New Zealand white female rabbits, weighing 3.5–4.5 kg (mean, (4.1±0.3) kg) were used in this study. Radiographs of both femorotibial joints were taken and evaluated by two orthopedists to exclude animals with any joint disease. Six rabbits were treated as controls, and the remaining 12 rabbits were assigned to the experimental group. Surgical transection of the anterior cruciate ligament in the left femorotibial joint was performed in 12 experimental rabbits under general anesthesia to induce OA. Routine skin incision closures were performed. Antibiotics (penicillin 20000 IU) were injected intramuscularly twice per day, prior to surgery and for 2 d postoperatively. After surgery, the rabbits were permitted free movement in their separate cages for the duration of the experimental period. Experiments on the rabbits were approved by our laboratory animal association and performed under the guidelines of the National Institute of Health for the care of laboratory animals.

At 2, 4, and 6 weeks after surgery, six animals were euthanized, including four experimental rabbits and two control rabbits. Each of the left knees was dissected and sectioned with a band saw to obtain the articular cartilage samples, with pathological characteristics. Overall, 72 cartilage specimens were collected, comprising samples from 18 medial femoral condyles, 18 lateral femoral condyles, 18 medial tibial plateaus, and 18 lateral tibial plateaus. Each sample was divided into two parts, one for pathological analysis and one for the swelling experiment. The average diameter of the samples was about 6 mm, and the average thickness was about 0.8 mm. All specimens were wrapped in wet gauze soaked with saline buffer and stored at −20°C until the ultrasound examination.

1.2 Pathological grade

According to the standards of OARSI, OA was graded as follows: Grade 1=uneven surface that can demonstrate superficial fibrillation; Grade 2=surface discontinuity that may be accompanied by cell proliferation, and increased or decreased matrix staining in the mid zone; Grade 3=vertical fissures extending into the mid zone or erosion; Grade 4 =denudation (the unmineralized hyaline cartilage is completely eroded); and Grade 5=deformation. OA develops over three stages: early stage (Grades 1–2), intermediate stage (Grade 3) and advanced stage (Grades 4–5) [17].

Cartilage samples used for pathological observation were fixed in 10% neutral buffered formalin, decalcified with 14% ethylenediamine tetra-acetic (EDTA) acid, dehydrated through graded alcohol solutions, cleared with toluene, and embedded in paraffin. Six-micrometer sections of articular cartilage were cut from the central part of the ultrasound-

observed sites. The sections were stained with Safranin O, and graded by three board-certified pathologists, blinded to the purpose of the study.

1.3 Ultrasound measurements

Figure 1 shows a high-frequency ultrasound experiment system. The system comprises one pulse transmitter/ receiver (Model 5900, Olympus, USA), a 50 MHz focused ultrasound transducer (Panametrics, USA, the focus was 12.7 mm), one computer with a 12-bit A/D converter and 400 MHz sample frequency (CompuScope 12400, Gage, Canada).

For measuring the swelling-induced strains of the cartilage tissue, a zero-swelling reference configuration was defined at 2 mol L^{-1} NaCl, as the charge shielding was effective at hypertonic ion concentrations and all swelling effects could be neglected [11,18,20]. During the free-swelling experiment, samples were first equilibrated for 1 h in 2 mol L^{-1} NaCl solution. The 2 mol L^{-1} saline solution was removed, and replaced with a physiological (0.15 mol L^{-1}) saline solution. The cartilage specimen was monitored with the ultrasound system for 2 h, during which the cartilage reached an equilibrium state. As the ion concentration inside the cartilage was higher than that of the external bathing solution, a Donnan osmotic pressure was generated. With the diffusion of the ions and water, the dynamic deformation of the cartilage layer at different depths could be observed from the ultrasound echo. All ultrasonic echo signals were sampled by the AD card and then stored in the computer. Figure 2 shows representative ultrasound echo signals of a cartilage sample at two different time points. The abscissa indicates the thickness of cartilage and the vertical axis represents the amplitude of the echo signal. The white triangle points to the AC surface, while black triangle points to the cartilage-bone interface.

The transient displacement of the AC tissue at selected depths during swelling was extracted, based on a cross-correlation echo tracking method using a custom-designed computer program developed with Mathematica (Ver 7.0, Wolfram Co., Champaign, IL, USA). The displacement difference of the tissue at every two adjunct depths was used to calculate the strain of the sublayers of the articular cartil- age, as previously described [16,18].

1.4 Biochemical analysis

To compute the uniaxial modulus of the cartilage based on the triphasic model, two important parameters needed to be obtained: the volume fraction of water (ϕ_0^{w}) and the density of the fixed charge (c_0^{F}). The full-thickness cartilage layer was serially sectioned into four layers, with a thickness of approximately 0.2 mm (Figure 3).

The slices were soaked in 2 mol L^{-1} saline solution for 1 h and the wet weight (WW_0) was measured. The slices were then soaked in 0.15 mol L^{-1} saline solution for 1 h, and cooled to −50°C before being freeze-dried for 48 h. The dry weight (DW) of each layer was measured. The ϕ_0^{w} in the reference configuration was calculated by eq. (1) [12]:

$$\phi_0^{\mathrm{w}} = \frac{WW_0 - DW}{(WW_0 - DW) + DW(\rho_{2\mathrm{M}} / \rho_{\mathrm{s}})}, \quad (1)$$

where ρ_{s} refers to the density of the cartilage solid matrix (1.323 g mL^{-1}), and $\rho_{2\mathrm{M}}$ denotes the density of the 2 mol L^{-1} NaCl solution [21]. To calculate the fixed charge density

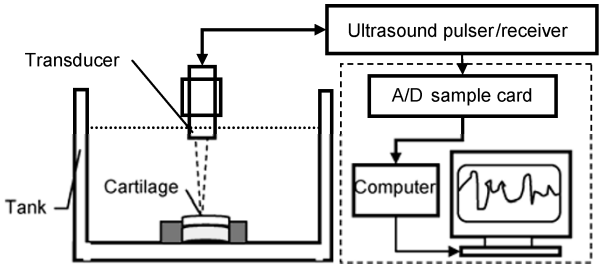

Figure 1 Schematic of the transient ultrasound measurement system.

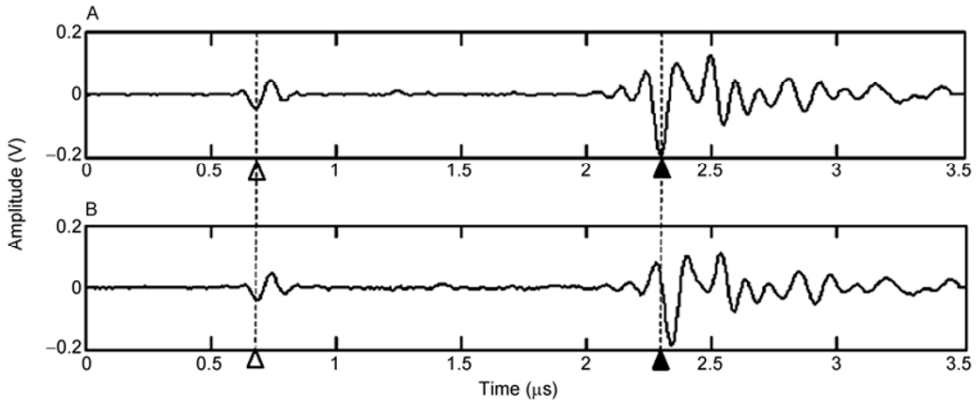

Figure 2 Ultrasonic echo signal of cartilage sample at different time points. A, At the beginning of the concentration change. B, At the balance condition. △, Cartilage surface; ▲, bone-cartilage interface.

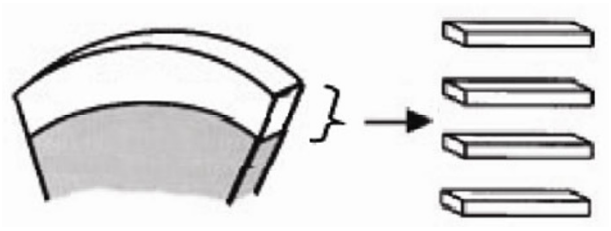

Figure 3 Schematic representation of slice preparation for biochemical analysis.

(FCD), an indirect method of image processing was designed to obtain the values of c_0^F based on the literature values, as previously described [20].

1.5 Calculation of the elastic modulus

In the triphasic model, cartilage was taken as a mixture of linear, isotropic, incompressible solid matrix and incompressible liquid matrix, including ions [11,12,19,22]. The fundamental concept of the triphasic theory of AC is the principle of a balance of forces. In the free-swelling experiment, the interstitial fluid pressure (p) approximately equaled the Donnan osmotic pressure, which is related to the content of fix charge density (c_0^F), water volume fraction (ϕ_0^w), ion concentration (ϕ_0^w) and the infinitesimal swelling strain tensor (E). The elastic stress on the cartilage solid matrix depended on the material properties and mechanical properties of the uniaxial modulus (Ha) and Poisson's ratio (v_s). In the balanced state, the sum of all forces inside the tissue was zero. The swelling strains of the cartilage could be predicted with biochemical, material, mechanical and geometric parameters of cartilage thickness [11,12,19,22]. According to the triphasic theory, the total stress in the isotropic porous-permeable mixture of the fluid, solid and ion phases in cartilage can be written as follows:

$$\sigma = \sigma^s + \sigma^w + \sigma^+ + \sigma^- = -pI + \lambda_s tr(\varepsilon)I + 2\mu_s\varepsilon, \qquad (2)$$

where σ represents the total stress for the mixture; the indices w, s, + and − denote quantities associated with water, solid, cation and anion, respectively; E is the infinitesimal strain tensor measured from the physiological salt bath corresponding to the hypertonic reference configuration; p is the fluid pressure; in this paper, $p=p_{Donna+}p_0-T_1$, p_{Donna} and p_0 are the Donnan and the entropic components of the swelling pressure in the fluid phase, respectively, where $T_1 = c_0^F kTN_{Avo}/1.5(1-\phi_0^w)\lambda_s$ and μ_s in eq. (2) are the Lame coefficients of the solid matrix. According to eq. (2), the total stress in cartilage in equilibrium under free-swelling conditions consists of two components: p and the elastic stress, which depends on the material properties of the solid matrix.

It was assumed in the model that the contribution of the entropic effects to cartilage swelling pressure was zero, and there was no external hydrostatic pressure [11]. Therefore, p approximately equaled p_{Donnan}, suggesting that all swelling effects were from the electrostatic interaction between negatively charged PGs and ions. A linear constitutive expression for p_{Donnan} can be obtained from the boundary conditions for the equivalent chemical potential of water and NaCl ions across the free surface:

$$p_{Donnan} \approx RT([(c_0^F)^2 + (2c^*)^2]^{1/2} - 2c^* - tr(E)\cdot(c_0^F)^2 / (\phi_0^w[(c_0^F)^2 + (2c^*)^2]^{1/2})), \qquad (3)$$

where R is gas constant, T is the absolute temperature and c^* is the NaCl concentration. c_0^F and ϕ_0^w of cartilage can be measured using a biochemical method. Poisson's ratio is set to a constant of 0.35. The relationship between uniaxial modulus (H_A) and the material parameters of articular cartilage can be described as

$$H_A=\lambda_s+2\mu_s.$$

In general, articular cartilage was considered as a multi-phasic mixture with an inhomogeneous structure. According to its structure, articular cartilage can be roughly divided into three zones: surface, middle and deep, occupying approximately 10%–20%, 40%–60%, and 30% of the total tissue thickness, respectively. The composition and structure vary with the different depths of cartilage. Therefore, the strains of each layer in the swelling process are different. In previous research, a modified two-layer model with four parameters was proposed [11,16,18,20]. In this inhomogeneous model (Figure 4), three moduli (Ha_1, Ha_2, Ha_3) were defined to describe the two-layer structure. In this paper, the modulus of AC was estimated based on this model.

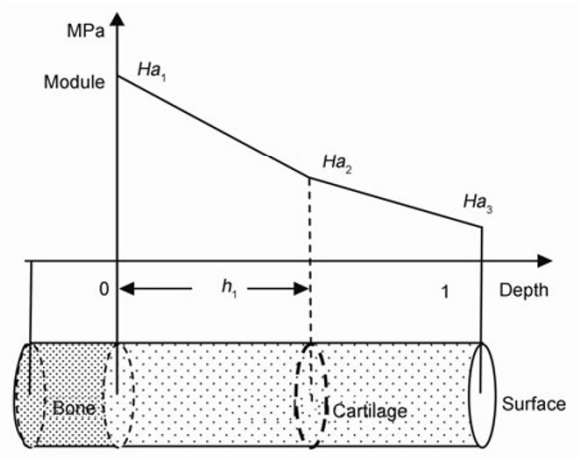

Figure 4 Schematic diagram of inhomogeneous two-layer triphasic model of articular cartilage. Three moduli: Ha_1, Ha_2, Ha_3. Cartilage thickness has been normalized.

1.6 Statistical analysis

Statistical analyses were conducted with SPSS software (Version 17, SPSS Inc., USA). All values in the text were presented as mean±standard deviation (SD). The analysis of variance (ANOVA) in the uniaxial modulus between the OA grades was performed using Pearson's correlation analysis. $P<0.05$ was taken as statistically significant.

2 Results

2.1 Pathological grade of cartilage samples

This study evaluated 72 articular cartilage samples according to the OARSI grading system. As listed in Table 1, 29 samples were normal, while 43 cartilage samples were abnormal, including 14 Grade 1 cartilage samples, 23 Grade 2 cartilage samples, and 6 Grade 3 cartilage samples. There were no Grade 4 or Grade 5 cartilage samples. Figure 5 shows the pathological, Safranin-O stained sections of normal versus Grades 1–3 OA cartilage. The normal cartilage appears flat and smooth, with homogeneous staining (Figure 5A). By comparison, the surfaces of the Grade 1 cartilage samples were uneven, with slight fibrillations (Figure 5B). In the Grade 2 cartilage samples, the Safranin O staining was fainter, and the cartilage surface appeared uneven (Figure 5C). In the Grade 3 cartilage samples, an obvious cartilage thinning was observed, with lighter staining in the surface and middle layers, and obvious pathological changes in the deeper layers of the tissue.

2.2 Uniaxial modulus of cartilage samples at different levels

Two parameters, c_0^{F} and ϕ_0^{w}, were obtained from biochemical analyses, and the second order curve of the parameters is shown (Figure 6). The abscissa indicates the four sliced layers from Figure 3. c_0^{F} and ϕ_0^{w} varied with the depth of the cartilage. As the depth increased (from the cartilage surface to the base), the c_0^{F} increased, but ϕ_0^{w} decreased. The c_0^{F} of the normal cartilage was higher than that of the OA cartilage, whereas the ϕ_0^{w} was less in normal cartilage.

Table 1 lists the uniaxial elastic modulus of normal cartilage and OA cartilages, calculated with the triphasic model. It can be seen that, for the cartilage of the same grade, the uniaxial elastic modulus in the surface layer was lower than

Table 1 The value of uniaxial moduli of articular cartilage with different OARSI grades[a)]

OARSI grade	Normal (29)	Grade 1 (14)	Grade 2 (23)	Grade 3 (6)
Ha_1	26.51±10.63	25.69±9.74	25.40±10.29	18.58±9.61
Ha_2	14.97±7.34	12.62±6.19	12.07±7.72	7.06±4.73
Ha_3	5.98±3.37	3.53±2.72	0.92±0.69	0.11±0.06

a) Measurements are the mean±SD. OARSI is the Osteoarthritis Research Society International grading system that is used to define the different grades of OA (Grades 1–5, with 5 being the most severe). Ha_1, Ha_2 and Ha_3 are the three moduli to describe the two-layer structure (Figure 4).

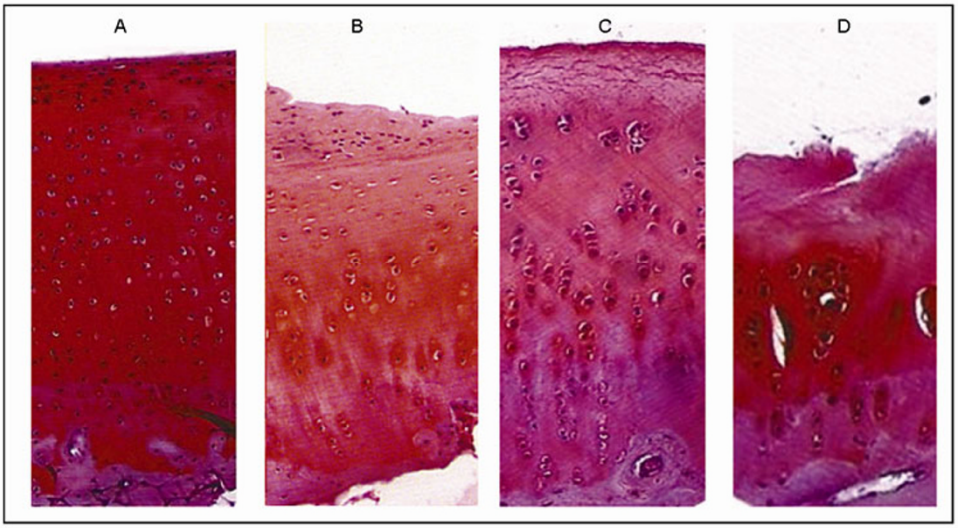

Figure 5 Images of safranin-O stained histologic sections to show cartilage formation. A, Normal. B, Grade 1. C, Grade 2. D, Grade 3.

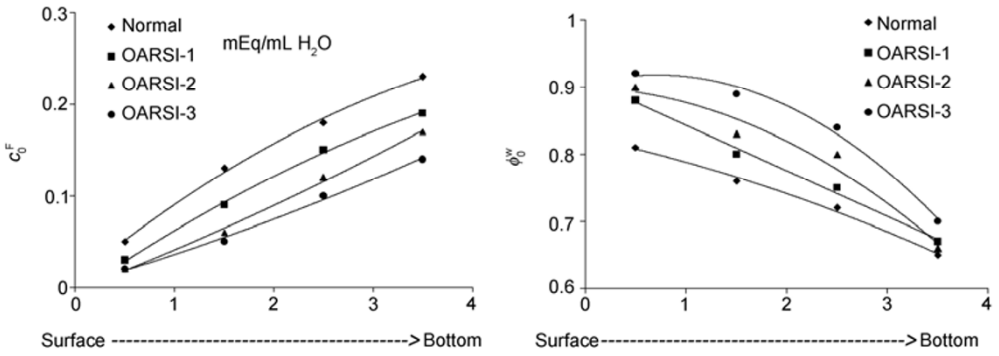

Figure 6 The average depth-dependent distribution of fixed charge density and water volume fraction of rabbit articular cartilage. ORSI Grades 1–3 are represented by the square, triangle and circle points, with normal tissue represented as diamonds. c_0^{F} represents the fix charge density.

those in the middle and deep layers. No significant differences were found in the deep layer among the normal cartilage and the Grades 1 and 2 OA cartilages (P<0.01). With an increase in OA grade, the unaxial elastic modulus showed a decreasing trend. Changes on the surface were the most obvious. The results of the statistical analysis showed that the unaxial elastic modulus of the surface layer was significantly different between the normal and OA cartilage with different grades (P<0.01). No significant differences were found in the middle layer between Grades 1 and 2 OA cartilages (P<0.01). The elastic modulus in the middle layer of Grade 3 was significantly lower than those of Grades 1 and 2 (P<0.05). Beyond Grade 3, the elastic modulus of the deep layer was significantly reduced.

3 Discussion

The unique composition and structure of AC determine its distinct biomechanical properties. The degeneration of cartilage tissue begins with changes in its composition and structure, and eventually results in OA. At present, pathological testing is commonly used by clinicians when grading OA. This paper studied the layered triphasic mechanical properties of OA cartilage with different pathological grades.

The pathological tests showed an obvious difference between the normal and the different grades of OA cartilage. The matrix of normal cartilage stained uniformly, and the surface was smooth. With the progression of OA, the cartilage surface became increasingly uneven, with a more obvious surface fibrosis. Previous studies have shown that, in the early stages of OA, the surface PGs change first, and their concentration in the deeper layers reduces with progressive cartilage degradation [1,17,18]. This hypothesis has been confirmed with biopsies. In this study, with the development of OA, Safranin O staining gradually reduced in intensity from the surface layers to the bottom layers, and sometimes the tissue did not stain at all. The cartilage in these layers also became thinner with progressive OA. These pathological results indicate the effectiveness of the animal OA model in this study.

The fixed charge density c_0^{F} increases with an increased tissue depth for both normal and OA cartilages, while the water volume fraction ϕ_0^{w} decreases, consistent with previous findings [12,16,18,19,22]. Compared with the OA cartilage, the c_0^{F} of normal cartilage was higher at the same tissue depth, but ϕ_0^{w} was lower. As OA progressed, these important parameters changed. The c_0^{F} value was closely related to the PG content, which was less on the surface of the cartilage, as compared with the deeper tissue layers, but ϕ_0^{w} was greater. With the development of OA, the PG content gradually decreased, and was replaced by water, which resulted in a lower value for c_0^{F} and a higher value for ϕ_0^{w}.

Using approaches such as confined compression, unconfined compression and indentation, other researchers have computed the elastic modulus of rabbit cartilage [23,24]. Korhonen *et al*. found that the elastic modulus varied with different approaches [25]. In addition, one common shortcoming of these studies is that the cartilage tissue is simply assumed to be homogeneous, and the estimated modulus is examined as a bulk property. However, in reality, the various grades of OA cartilage will degrade at different rates and to different depths. Thus, this homogenous assumption is oversimplified.

The statistical results showed a lower uniaxial elasticity modulus in the surface layer than in the middle layer for the same grade of samples, and a lower modulus in the middle layer as compared with that in the deep layer. These results indicated that the material properties of cartilage tissue change along with tissue depth. No significant difference was found in the deep layer among the normal cartilage and the OA cartilages of Grades 1 and 2 (P<0.01), indicating an unchanged tissue composition and structure in the deep lay-

er of OA cartilage. With an increase in the grade, the uniaxial elastic modulus of the cartilage surface and middle layers showed a gradual decreasing trend. Changes to the surface layer were the most obvious. The results indicate that when OA occurs, the modulus for the surface layer is the first to change. The statistical results showed that the uniaxial elastic modulus on the surface of normal cartilage was quite different from that of OA cartilage (P<0.01). There was a clear difference in surface elastic modulus among OA cartilages with different grades (P<0.01), which coincided with the pathological results of the cartilage biopsy. As OA progressed, the composition and structure on the surface shifted toward a lower elastic modulus. In the middle layer, no significant difference was found in the elastic modulus between OA Grades 1 and 2 (OARSI) (P<0.05), indicating that in the early stages of OA, the main damage occurs in the surface layer. The elastic modulus of the Grade 3 OA cartilage in the middle layer was significantly lower than that in Grades 1 and 2 (P<0.05), showing that an intermediate OA significantly affects the tissue in the middle layer. Meanwhile, the elastic modulus in the deep layer of Grade 3 OA cartilage was lower. We conclude from these results that the elastic modulus estimated by the triphasic theory can quantitatively describe the pathological conditions of cartilage and can be used to grade the severity of OA.

This study did not assess OA cartilage samples of Grades 4 or 5. According to the classification of OARSI, cartilage with Grade 4 or 5 is highly degenerated. The cartilage tissue and subchondral bone both show signs of damage, and the tissue thickness is reduced. As such, it would be difficult to measure the mechanical parameters of Grade 4 or 5 tissue samples in swelling experiments. Furthermore, these two grades are considered as severe OA. In these cases, the clinical symptoms are obvious, so there is little clinical benefit in obtaining these parameters for diagnosing OA. One limitation of this study is the small and uneven tissue sizes of our samples. In particular, the sample size of Grade 3 is noticeably smaller. Future studies should use larger tissue sample sizes.

In summary, this study explored the differences in the layered elastic modulus among cartilage samples with different grades of OA. It provided a new method to quantitatively evaluate the severity of OA using the mechanical properties of the cartilage tissue. While this study confirms the use of non-contact ultrasonic measurements and the triphasic model to assess OA, further study is needed before this technique can be applied in the clinical setting.

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