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Physical mechanisms underlying the strain-rate-dependent mechanical behavior of kangaroo shoulder cartilage

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Due to anatomical and biomechanical similarities to human shoulder, kangaroo was chosen as a model to study shoulder cartilage. Comprehensive enzymatic degradation and indentation tests were applied on kangaroo shoulder cartilage to study mechanisms underlying its strain-rate-dependent mechanical behavior. We report that superficial collagen plays a more significant role than proteoglycans in facilitating strain-rate-dependent behavior of the kangaroo shoulder cartilage. By comparing the mechanical properties of degraded and normal cartilages, it was noted that proteoglycan and collagen degradation significantly compromised strain-rate-dependent mechanical behavior of the cartilage. Superficial collagen contributed equally to the tissue behavior at all strain-rates. This is different to the studies reported on knee cartilage and confirms the importance of superficial collagen on shoulder cartilage mechanical behavior. A porohyperelastic numerical model also indicated that collagen disruption would lead to faster damage of the shoulder cartilage than when proteoglycans are depleted. © *2015 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4929498]

Articular cartilages, predominantly a "mechanical" biotissue, have the ability to endure a lifetime of varying physiological strain-rates without any significant damage. The superior mechanical properties and behavior of cartilages are known to be due to the structural make up, organization and properties of the constituents which are water swallowing proteoglycans and the collagen network.[1,2] The early stage of osteoarthritis is characterized by degradation of superficial collagen and proteoglycans which subsequently lead to severe proteoglycan loss and collagen disruption.[3–7] Therefore, investigations into the role of proteoglycans and the collagen network on the strain-rate-dependent response are important for understanding tissue behavior in osteoarthritis sufferers, the development of strategies for early stage diagnosis of the disease, and development of engineered cartilage tissues.

The dynamic properties of cartilages (extracted at high strain-rates) are considered to be governed by the structure of the collagen network.[8,9] Based on a finite element (FE) model that considered the cartilage structure and composition, Julkunen *et al.*[10] showed that superficial collagen can considerably affect tissue behavior at high strain-rates, i.e., 10^{-1}/s or larger. In contrast, the equilibrium properties of cartilages (extracted at zero strain-rate) are known to be mainly affected by the proteoglycans.[8,11] It is also well accepted that the compressive properties of cartilages are directly affected by the proteoglycans. However, conclusions of most studies do not consider that the proteoglycan composition and the structural features of the collagen network adapt to external mechanical stimuli, and hence, depend on the local mechanical environment of the tissue.[12–18]

Chondrocytes dynamically synthesize the extracellular matrix (i.e., proteoglycans and collagen) based on the external loading stimuli they receive.[19–21] For example, the proteoglycan content of knee cartilages, which bear high compressive loads, is higher than upper limb cartilage tissues which experience less compressive loading.[15,22,23] Differences in the collagen architecture of knee and upper limb cartilages have also been reported.[12] The conclusions of reported studies,[8–11] predominantly for knee cartilages, should therefore be evaluated in the context of the tissue studied. As shoulder cartilages experience considerably less compressive loading, we hypothesize that the collagen network (including the superficial layer) may play a more significant role in facilitating strain-rate-dependent behavior of the shoulder cartilage than proteoglycans.

Indentation tests on cartilage have been widely used to obtain mechanical properties of tissues due to the simplicity and potential for use in clinical diagnosis of tissue related diseases.[24–26] In addition, artificial degradation through enzyme treatment is commonly used to model proteoglycan loss and superficial collagen damage.[8,27] The main advantage of the artificial degradation is that the level of damage to the tissue can be controlled through enzyme concentration, the type of enzyme used and duration of the exposure.[27,28] Hence, artificial degradation can also be used to understand the role of individual constituents on mechanical behavior of the tissue.

The present study uses mechanical indentation testing along with controlled enzymatic degradation to investigate the role of superficial collagen and proteoglycans on strain-rate-dependent behavior of the shoulder cartilage. Degradation of the proteoglycans and collagen will result in an increase of pore size and weakening of the tissue's structural integrity, respectively. These effects are expected to significantly compromise the tissues' ability to respond to

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different strain-rates. Comparing the effects of proteoglycan and superficial collagen degradation will contribute to understanding the physical mechanisms and constituents responsible for the strain-rate-dependent mechanical behavior of shoulder cartilage.

The kangaroo has been recently proposed as a suitable animal model to explore the mechanical behavior of the human upper limb cartilages.[12,15] Therefore, visually normal (ICRS[29] macroscopic score = 0) cartilage samples of 8 mm diameter with 2–3 mm of subchondral bone intact were carefully harvested from the central load bearing area (Fig. 1(a)) of the humeral head of adult red kangaroos (≈5yr old) using a custom-made stainless steel puncher, within 24-h of slaughter. The ethical clearance for using kangaroo cartilage tissue was obtained from Research Ethics Unit of Queensland University of Technology (Approval No: 1200000376). In the experimental design stage, it was noticed that testing on a sample would take 1–3 days to complete. Therefore, the experimental procedure had to be designed in order to reduce the possible effects of tissue preservation. One method of sample preservation was to freeze samples in phosphate-buffered saline (PBS) inhibitor solution supplemented with antibiotics (200 mM L-glutamine, 10 000 units of Penicillin and 10 mg/ml of streptomycin; Sigma-Aldrich, Castle Hill, New South Wales) and to thaw samples in the PBS for approximately 30 min before mechanical testing. A second method was to preserve samples in a PBS-inhibitor solution at 4 °C until experimentations are completed. After assessing the two methods and considering that multiple freeze-thaw cycles may affect the tissue structure,[30,31] the second method was chosen to preserve the tissues (see Sec. 1 of the supplementary material[32]). Individual cartilage thickness was calculated using the average ultrasound speed in the kangaroo shoulder cartilage, measured to be 1658.3 ms^{-1} (see Sec. 2 of the supplementary material[32]).

During mechanical testing, the subchondral bone of harvested samples was constrained using a stainless steel holder and was indented up to 25% engineering strain (Fig. 1(b)). A safe limit of 3.5 MPa for strain-rates between 3×10^{-5}/s and 7×10^{-1}/s has been suggested to prevent damage to the cartilage matrix.[33,34] Therefore, in the present study a limit of 3.0 MPa was imposed on the maximum stress that samples were subjected to in order to reduce potential tissue damage. The strain-rates of the present study were chosen to be 10^{-4}/s, 5×10^{-4}/s, 5×10^{-3}/s, and 10^{-2}/s considering the reported physiological strain-rates experienced by cartilages.[33–35] The testing was done using a high resolution Instron testing machine (Model 5944, Instron, Canton, Massachusetts, USA) using a plane-ended, polished indenter of 3 mm diameter with rounded edge of 0.1 mm radius. An indenter with rounded edge was chosen to reduce possible local damage to the cartilage due to stress concentration at the indenter edges. After each test, the cartilage was allowed to recover for 1 h in the PBS-inhibitor solution prior to the next test.

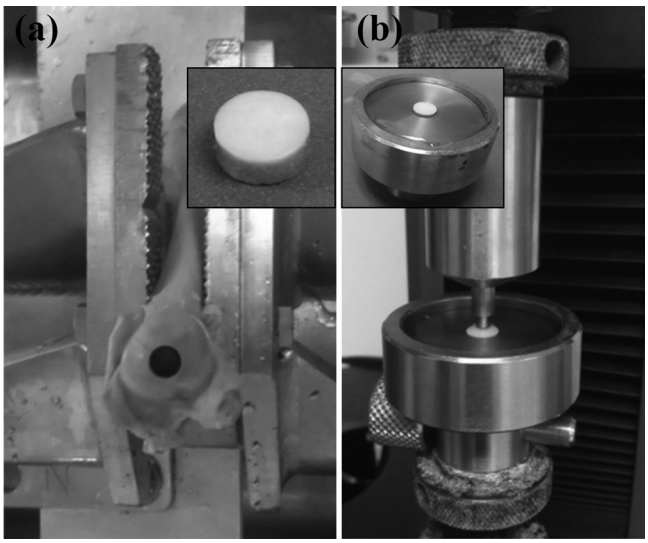

FIG. 1. (a) The sample harvested from central load bearing area of the humeral head of an adult red kangaroo (b) Indentation testing is conducted after the sample is constrained in a sample holder.

In this study, we tested two sample groups. In the first group (n = 12), proteoglycans were progressively degraded for 1 h, 2 h, and 4 h and in the second group (n = 10) collagen was degraded for 44 h. Proteoglycans were degraded using 0.05 mg/ml Trypsin-PBS solution and collagen was degraded using a 30 U/ml collagenase solution (see Secs. 3 and 4 of the supplementary material[32]). The protocols used for the constituent degradation is in accordance with the previous studies.[27,28,36] The 4 h Trypsin-PBS treatment and 44 h collagenase treatments are known to remove all the proteoglycans and significantly disrupt superficial collagen, respectively. The alcian-blue test (see Sec. 5 of the supplementary material[32]) indicated that collagenase treatment removed only a small amount of proteoglycans from the tissue matrix, similar to the previously reported findings.[28,37] After each enzymatic treatment, samples were subjected to indentation testing at the above mentioned four strain-rates.

In order to investigate the strain-rate-dependent mechanical properties of kangaroo shoulder cartilage, Young's modulus was extracted from force-indentation curves. The behavior of the kangaroo shoulder cartilage can be represented by 2-term reduced polynomial hyperelastic function.[38] In the present study, the relationship between force (F) and indentation depth (δ) given by Lin *et al.*[39] for the 2-term reduced polynomial hyperelastic model was modified to account for indenter geometry and finite sample thickness. Other methodologies, such as by Zhang *et al.*,[40] can also be applied to obtain force-indentation relationship for hyperelastic materials. The modified relationship is given by the following equation (see Sec. 6 of the supplementary material[32]):

$$F = 2\pi k_1 C_{10}\left(\frac{\delta^3 r - 3\delta^2 r^2 + 3\delta r^3}{\delta^2 - 2\delta r + r^2}\right) + 2\pi k_2 C_{20}\left(\frac{\delta^3 r - 3\delta^2 r^2 + 3\delta r^3}{\delta^2 - 2\delta r + r^2}\right)\left(\frac{3\delta^2 r - \delta^3}{r^3 - \delta r^2}\right), \quad (1)$$

where r is the indentation radius, C_{10} and C_{20} are the hyperelastic material constants. C_{20} is a nonlinear stiffness parameter. C_{10} is related to Young's modulus (E) and Poisson's ratio (ν) via the following equation:

$$C_{10} = \frac{E}{3\pi(1-\nu^2)}. \quad (2)$$

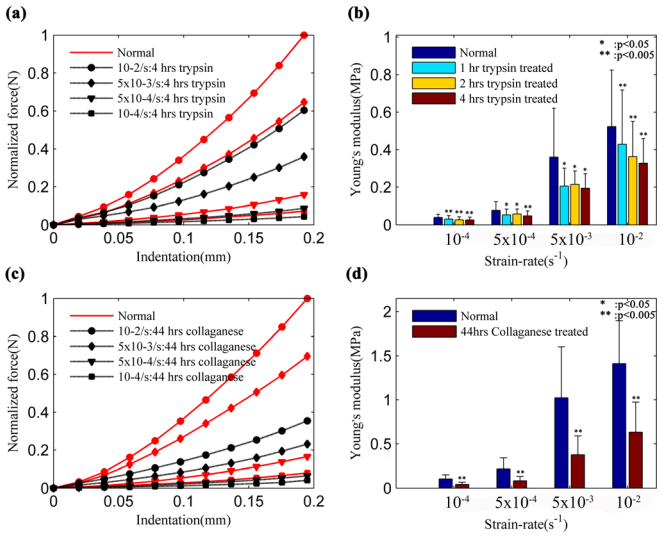

FIG. 2. (a) The average (n = 12) normalized force-indentation curves for samples treated in trypsin for 4 h. (b) Young's modulus variation with 1 h, 2 h, and 4 h trypsin treatment for four strain-rates. (c) The average (n = 10) normalized force-indentation curves for samples treated in collagenase for 44 h. (d) Variation of Young's modulus with strain-rate for collagenase treated samples.

In Equation (1), k_1 and k_2 are the factors that account for the indenter geometry and the finite thickness of the tissue and are related to thickness (h) over r via Equations (3) and (4) (see Sec. 6.1 of the supplementary material[32])

$$k_1 = 2.306\left(\frac{h}{r}\right)^{-1.568}, \tag{3}$$

$$k_2 = 0.958\left(\frac{h}{r}\right)^{-5.43}. \tag{4}$$

In order to obtain Young's modulus, a computer program was developed using Matlab R2014a (The MathWorks, Inc.) to solve the nonlinear least-square minimization problem of curve-fitting the force-indentation data to Equation (1). Since Equation (1) has been derived assuming material incompressibility, Poisson's ratio was taken to be 0.5 in the present study. The effect of enzymatic degradation on permeability of the tissue was also assessed and was extracted by curve fitting a porohyperelastic model to experimental force-indention curves at the smallest stain-rate (10^{-4}/s) using inverse finite element analysis.[41]

The mechanical behavior and properties of normal, proteoglycan degraded and collagenase degraded tissues were statistically compared with each other. The Repeated measure analysis of variance (ANOVA) was used to identify the statistical significance of the treatments while Tukey's pairwise comparison test was employed to compare between the individual levels of treatments. Minitab version 16.1.1 (2010 Minitab Inc.) was used for the statistical analysis. In this study, the statistical significance is reported at both 95% ($p < 0.05$) and 99.5% ($p < 0.005$) confidence intervals.

Fig. 2 presents the average normalized force-indentation curves for two sample groups tested: trypsin treated (Fig. 2(a)) and collagenase treated (Fig. 2(c)). For the purpose of comparison between the two treatments, the force-indentation curves are normalized. The mean thickness of samples was 0.76 ± 0.16 mm and 0.78 ± 0.10 mm for the proteoglycan and collagenase treated groups, respectively ($p = 0.681$). Although samples were harvested from randomly picked shoulder joints and tight control on experimental procedure was employed, average Young's modulus (Table I) was observed to be different in these two groups ($p < 0.05$) which can be attributed to inherent biological variation of samples. It was found that the stiffness of the kangaroo shoulder cartilage increases with strain-rate, which has been reported previously for other cartilage tissues.[35,42] The strain-rate-dependent behavior was still a characteristic of the tissue even after the proteoglycan and collagen degradation (Figs. 2(a) and 2(c)). However, the proteoglycan and collagen degradation significantly compromised the ability of tissue to respond to varying strain-rates resulting in tissues less capable of withstanding external loads ($p < 0.05$).

Young's modulus at all strain-rates reduced gradually (Fig. 2(b)) with the progressive removal of proteoglycans, and is statistically significant for 1 h, 2 h, and 4 h trypsin treatments when compared with normal tissue ($p < 0.05$). These results confirm the already established knowledge that proteoglycans have a direct role in compressive load bearing of cartilage tissues. Permeability increased from 1.38 ± 0.8310^{-14} m^4/Ns to $3.03 \pm 1.43 \times 10^{-14}$ m^4/Ns due to the proteoglycan degradation ($p < 0.005$), which is similar to previously reported studies.[8] The permeability values correspond to a pore size of 160.38 ± 37.49 Å and 238.96 ± 48.00 Å, respectively, which represents an increase of 1.48 times.

It was believed that complete removal of proteoglycans would increase the pore size of the tissue to an extent that the solid-interstitial fluid frictional interactions would be considerably reduced, which is one of the main contributors to the strain-rate-dependency of cartilage tissues.[43,44] Hence, the complete removal of proteoglycans was expected to almost completely remove the strain-rate-dependent nature of cartilage. However, even after 4 h of trypsin treatment the strain-rate-dependent behavior can still be observed. This implies that the dense collagen network still sustains the size of pores in cartilage to an extent that solid-interstitial fluid frictional interaction is able to facilitate the tissues ability to respond to varying strain-rates or it may be due to the flow-independent

TABLE I. Young's moduli (MPa) of 4 h trypsin treated and 44 h collagenase treated kangaroo shoulder cartilage at four strain-rates.

Strain-rates	10^{-4}/s	5×10^{-4}/s	5×10^{-3}/s	10^{-2}/s
0 h in trypsin (n = 12)	0.040 ± 0.016	0.078 ± 0.445	0.360 ± 0.261	0.523 ± 0.299
4 h in trypsin	0.026 ± 0.014	0.048 ± 0.026	0.194 ± 0.076	0.328 ± 0.131
0 h in collagenase (n = 10)	0.10 ± 0.045	0.217 ± 0.125	1.023 ± 0.580	1.412 ± 0.485
44 h in collagenase	0.043 ± 0.025	0.084 ± 0.049	0.377 ± 0.214	0.633 ± 0.341

viscoelasticity of collagen network, which is reported[45,46] to contribute the strain-rate-dependency of cartilage.

The samples treated for 44 h in collagenase showed a significant decrease (Fig. 2(d)) in Young's modulus at all strain-rates ($p < 0.005$). The permeability values were $1.36 \pm 0.41 \times 10^{-14}$ m^4/Ns and $4.19 \pm 2.79 \times 10^{-14}$ m^4/Ns for normal and collagenase treated tissues, respectively. These permeability values correspond to pore sizes of 162.16 ± 22.32 Å and 270.22 ± 94.96 Å ($p < 0.05$), respectively, which is a 1.67 times increase in pore size. This permeability increase due to collagenase treatment is similar to previously reported studies.[8] Collagenase treatment for 44 h is known to significantly degrade the superficial collagen.[47] The results of the alcian blue experiment confirmed that a large portion of proteoglycans are still intact in the tissue matrix (Sec. 5 of the supplementary material[32]). Therefore, the decrease in the tissue stiffness and strain-rate-dependency as well as the increase in permeability is mainly due to the degraded collagen matrix.

In cartilage, water swallowing proteoglycans constrained by three-dimensional collagen network form the functional load-bearing unit of cartilage. Any disruption of the collagen network would reduce its ability to constrain the proteoglycans, compromising the matrix integrity and its ability to act as an effective load-bearing unit. In addition to the reduction in tissue stiffness, collagen disruption can increase the interspaces between collagen fibrils, i.e., the pore size and the permeability of tissue as observed in the present study. Therefore, the significant reduction in strain-rate-dependency observed, even more than the case of 4 h trypsin treated samples, confirms the importance of the collagen network in facilitating strain-rate-dependent behavior of cartilage.

Interestingly, when comparing the effect of 4 h trypsin treatment and 44 h collagenase treatment (Fig. 3(a)), it was noted that the collagenase treatment reduced tissue stiffness more at all strain-rates ($p < 0.05$). Therefore, in shoulder cartilage the superficial collagen contributes more than the proteoglycans when responding to compressive loads. This is understandable considering that chondrocytes can synthesize the extracellular matrix according to the mechanical inputs it receives. Hence, larger and more frequent the compressive forces, higher the proteoglycan composition and its role on the tissue behavior. Note that the shoulder joint experiences low magnitude compressive loads and therefore the stimulation of chondrocytes by compressive forces is also considerably low. Thus the amount of proteoglycans in shoulder cartilages is small, indicating that the collagen architecture

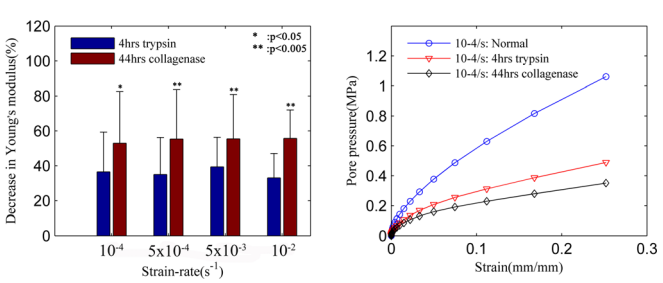

FIG. 3. (a) Percentage decrease in Young's modulus in 4 h trypsin treated and 44 h collagenase treated samples. (b) Variation of pore-pressure with strain for 4 h trypsin treated and 44 h collagenases treated samples.

of shoulder cartilage plays a dominant role in the tissue behavior as indicated by the results of the present study.

These findings are further reinforced by the observation that 44 h collagen degradation more or less had an equal ($p > 0.1$) effect on the tissue behavior at all strain-rates tested while similar observation can be made for 4 h proteoglycan degradation (Fig. 3(a)). The role of proteoglycans facilitating the tissue behavior equally even at different strain-rates has been reported earlier,[10] and is justifiable considering its direct role in facilitating compressive load-bearing ability of the tissue. However, the finding that superficial collagen affects the tissue behavior equally at all strain-rates is contrary to that reported in literature. On investigating the tissue behavior from 10^{-3}/s to 10^{-1}/s, Julkunen *et al.*[10] reported that superficial collagen only contributes to the tissue behavior substantially at the highest strain-rate, i.e., 10^{-1}/s. However, in their study, the contribution of proteoglycan (approximately 37.2%) on the tissue behavior at 10^{-1}/s was still much higher than the contribution of superficial collagen (14.7%). In comparison, by calculating the average percentage decrease in Young's modulus (Fig. 3(a)) at the strain-rates tested, the results of the present study indicated that the contribution of the superficial collagen to the tissue behavior of shoulder cartilage is $54.88 \pm 1.11\%$ while the contribution of proteoglycans is $35.99\% \pm 2.3\%$. The difference in observation of the present study with the reported studies is reasonable considering that the reported studies are for knee cartilages which are structurally and compositionally different from the shoulder cartilages. As mentioned, the collagen plays a dominant role in the mechanical behavior of shoulder cartilages, to an extent even larger than the proteoglycans. Therefore, an equally dominant effect of collagen on mechanical behavior of shoulder cartilage at all strain-rates is justifiable.

Another interesting observation is that, on average, the collagen disruption and proteoglycan degradation in total contributed to 89%–95% (Fig. 3(a)) reduction in total tissue stiffness. This implies that the total removal of proteoglycans and significant disruption of superficial collagen would render the shoulder cartilage almost incapable of responding to varying rates of external loads. Although dominated by the collagen network, this shows the important functional interdependency of collagen and proteoglycans in facilitating the strain-rate-dependent behavior of shoulder cartilage.

In order to understand the changes in the internal tissue behavior when the proteoglycans and collagens are degraded, a validated porohyperelastic FE model was employed (see Sec. 7 of the supplementary material[32]). Based on the FE model predictions, as shown in Fig. 3(b), for the strain-rate of 10^{-4}/s, the hydrostatic excess pore-pressure decreases considerably due to degradation of proteoglycan and collagen. The results are due to the decrease in elastic properties and increase in permeability when the tissue is degraded. The fluid is less capable of contributing to the load bearing function in the case of collagen degradation when compared with the proteoglycan degradation. Hence there will be more burdens on the collagen network when the superficial collagen is degraded, which will lead to the collagen network being further damaged and ultimately dysfunctional.

In summary, the present study investigated the physical mechanisms underlying the strain-rate-dependent behavior

of kangaroo shoulder cartilage. The results of the study revealed that proteoglycan depletion and superficial collagen disruption substantially compromised the tissues' ability to respond to different strain-rates. Superficial collagen was found to play a more important role than the proteoglycans in facilitating strain-rate-dependent behavior of the tissue and contributed evenly to tissue behavior at all strain-rates. This is in contrast to the conclusions reported on knee cartilages where the superficial collagen is reported to contribute less than proteoglycans to the mechanical behaviour, and the role of superficial collagen becomes substantial only at large strain-rates. Based on the porohyperelastic modelling, it was found that the collagen disruption would lead to shoulder cartilage being damaged faster than when the proteoglycans were depleted due to interstitial fluid being less capable of supporting external loads.

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