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The ionic contribution of proteoglycans to mechanical stiffness of the meniscus

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ABSTRACT

Load transmission is an important function of the meniscus. In articular cartilage, proteoglycans help maintain hydration via negatively charged moieties. We aimed to investigate the influence of electrostatic effects on stiffness of meniscal tissue.

Circular discs from bovine menisci of 8 mm diameter and 5 mm thickness were placed within a confined compression chamber. The apparatus was bathed in distilled water, 0.14 M PBS or 3 M PBS before being subjected to 5% ramp compressive strain and held for 300s. FEBio software was used to fit resultant relaxation curves to a non-linear poroviscoelastic model with strain dependent Holmes-Mow permeability. Analysis was conducted using one-way ANOVA with Tukey post-hoc analysis.

10 samples were tested in each solution. Significant differences ($p < 0.05$) were observed between the values for Young's modulus, zero strain dependent permeability and the viscoelastic coefficient for samples tested in 3 M PBS as compared to deionised water/0.14 M PBS. No significant differences were observed in the strain dependent/stiffening coefficients or the relaxation time. Approximately 79% of the stiffness of the meniscus appears attributable to ionic effects.

Ionic effects play a significant role in the mechanical stiffness of the meniscus. It is important to include the influence of ionic effects when developing mathematical models of this tissue.

1. Introduction

The menisci of the knee, historically thought of as vestigial remnants [1], are now understood to play a critical role in load transmission across the knee [2–4]. Histologically, the water content of the meniscus is estimated at 74% whilst the remaining dry weight is 75% collagen. Collagen fibres are arranged circumferentially in deeper layers of the tissue and tangentially in superficial layers [5]. Load transmitted to the meniscus is resisted by the firm ligamentous attachments of the menisci to bone, generating circumferential tensile (hoop) stresses in the aforementioned circumferential collagen fibres [5,6]. In addition, proteoglycans in the meniscus aid resistance against compressive loading of the meniscus. These large proteins are immobilised in the menisci by the collagen fibres [7] and aid fluid distribution, generating osmotic pressure gradients. Generation of such pressure is also aided by the low permeability of meniscal tissue [5]. Proteoglycans are highly anionic due to their attached glycosaminoglycans, which are present in a variety of connective tissues [8]. The predominant proteoglycan present in the meniscus is chondroitin sulphate, with a smaller proportion of dermatan sulphate also [9]. These types of proteoglycan complexes are similar to those observed in cartilage [10], though the concentrations of proteoglycans within meniscal tissue is lower [11] and has reported to be 1/8th of that in cartilage [12]. Study of porcine menisci suggests that there is a variable concentration of proteoglycans across the width tissue, with the inner surface of the meniscus, which is exposed to the highest loads, demonstrating the highest concentration of proteoglycans [13]. Proteoglycans are thought to be largely responsible for the viscoelastic properties of the meniscus in compression [14].

The mechanism through which proteoglycans mediate their effects has been studied in a number of other connective tissues. In articular cartilage and in the nucleus pulposus of the intervertebral disc, proteoglycans exhibit a fixed negative charge due to chondroitin sulphate and keratin sulphate molecules dissociating in solution. In turn, this leads to the development of a high Donnan osmotic pressure in the tissue [15]. This is due to the proteoglycans in the tissue being effectively trapped within the tissue due to their large molecular mass and leads to the tissue itself acting as a semi-permeable membrane. This osmotic pressure leads to

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an ability of the tissue to resist load. Fluid either exits the tissue (under load) or is re-imbibed (during unloading) to allow the osmotic gradient to reach equilibrium. This swelling is opposed by the collagen fibrillar network, which resists the tension generated by such swelling during loading of the tissue [16].

In articular cartilage, depletion of proteoglycans has been shown to result in a reduced compressive modulus [17]. Furthermore, increasing ionic concentration of bathing solutions results in reduced shear modulus of cartilage [18]. Korhonen and Jurvelin [19] tested bovine humeral articular cartilage in solutions of varying ionic concentrations in unconfined compression, fitting a poroelastic biphasic finite element model to demonstrate that an increase in osmolarity of the bathing solution resulted in a decrease in compressive modulus, whilst a decrease resulted in the opposite effect. The authors also showed a correlation between the proteoglycan concentration within the tissue and the compressive modulus measured, hence leading them to suggest that tissue proteoglycans account for a significant proportion of the compressive properties of articular cartilage. Notably, Canal Guterl et al. [15] suggest that the Donnan osmotic pressure induced by proteoglycans cannot account for the observed contribution of proteoglycans to tissue stiffness. Through testing of bovine articular cartilage in solutions of varied ionic strengths, coupled with mechanical testing of cartilage before and after proteoglycan digestion, they suggest that the combined electrostatic and non-electrostatic contribution of proteoglycans to the compressive modulus of articular cartilage accounts for >98% of the compressive modulus. Furthermore, ionic effects were thought to represent 62% of this total, with a decrease in the compressive modulus observed in solutions of high ionic concentration. Investigation into the effect of the fixed charge density of proteoglycans within articular cartilage during simulated walking in a finite element model of the knee suggests that the importance of this effect is amplified as the collagen network degenerates, in conditions such as osteoarthritis [20].

Proteoglycans are also understood to aid load transmission in the nucleus pulposus of the intervertebral disc, a tissue which withstands significant compressive load in the lumbar spine [21]. The structure of proteoglycans within the nucleus pulposus has been found to differ from that observed in other tissues such as articular cartilage or meniscus, with non-aggregated monomers of short length present [22]. However, testing of bovine nucleus pulposus in confined compression in physiological and hypertonic saline solutions suggests that, similar to articular cartilage, the stiffness of nucleus pulposus has both ionic and non-ionic components, with the ionic component responsible for 70% of the stress response [23].

The mechanism and extent by which proteoglycans exert this effect has not been demonstrated in meniscal tissue and the contribution of proteoglycans to the mechanical stiffness of the meniscus has not been previously quantified. Such data is important for a complete constitutive description of the tissue enabling appropriate models to be constructed and parameterised. This paper therefore describes a series of experiments to determine the magnitude of the ionic contribution of proteoglycans to mechanical stiffness of the bovine meniscus.

2. Methods

Bovine hind legs were obtained from a local abattoir immediately after slaughter. Menisci were excised and immediately frozen at −20 °C until the day of testing. Freezing meniscal tissue has been demonstrated to have no significant effect on ultrastructure of the tissue [24] or proteoglycan content [25]. All experiments were conducted within 6 weeks of slaughter. All animal was conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. A circular punch was used to obtain 8 mm diameter cylindrical cores of meniscus in the axial plane from the junction of the middle and outer third of the meniscus. A custom sectioning device was used to cut 5 mm thick discs of tissue from these samples. Both femoral and tibial surfaces were removed to generate a mid-substance sample. A micrometre was used to verify sample thickness. Discs were immediately wrapped in a non-permeable plastic film and allowed to defrost for 2 h.

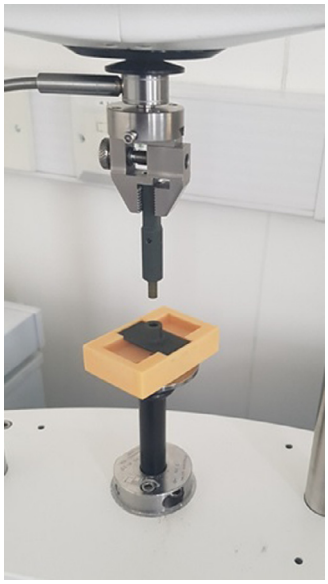

Fig. 1. Confined compression apparatus (bath allowing entire apparatus to be bathed in solution removed for clarity).

To investigate the influence of ionic effects, we chose to undertake confined compression experiments in solutions of varying ionic concentration. It has been demonstrated that whilst a 3 M PBS solution is sufficiently hypertonic to negate ionic effects in both articular cartilage [26] and intervertebral disc [23], whilst a deionised water solution negates all mobile ion effects.

Following defrosting, samples were placed into a confined compression chamber, permeable at the bottom only (pore size 400 µm) (Fig. 1). Samples were bathed in one of three solutions – deionised water, 0.14 M PBS or 3 M PBS and immediately subjected to a confined compression protocol as detailed below. The apparatus was placed within a Bose Electroforce 3100 materials testing machine and a custom waveform was created as follows. A porous indenter (pore size 400 µm) conforming to the dimensions of the compression chamber was used under load control to apply a preload of 0.3 N, followed immediately by a 5% ramp strain under displacement control at 1% per second, hence the total strain was applied over 5 s for each sample. The strain rate was chosen to allow the requisite strain to be applied without overloading the load cell and was arbitrarily chosen as a balance between the material testing machine's capabilities and requirements of the model used. Both the indenter and compression chamber were comprised of a plastic polymer with a flexural modulus of 3350 N/mm^2.

Separately conducted preliminary testing of samples had established that if subjected solely to a 0.3 N preload in 0.14 M/3 M PBS reached load equilibrium within 1–2 h, those tested in deionised water did not reach load equilibrium at > 6 h due to persistent swelling, with swelling effects becoming dominant at 300 s. Hence, to allow samples in each solution to be tested with similar preconditioning, we did not seek to test at load equilibrium, but instead applied a 0.3 N preload followed by an immediate ramp strain compression as detailed above. As swelling effects became

dominant at 300 s, relaxation data was collected for only this time period. Hence, each sample was tested over a ~5 s ramp phase and a 300 s hold phase. Each sample was tested only once, in one of the three solutions.

FEBio software [27] was used to develop a non-linear biphasic poroviscoelastic finite element model with strain dependent permeability [28]. The model consisted of 404 nodes, with 100 elements. A convergence study suggested a percentage error of <0.1% for a model with 404 nodes compared to one with ~1000 nodes, thus a 404 node model was chosen to allow an acceptable compromise between accuracy and numerical efficiency. Boundary conditions were set to confined compression and the Poisson's ratio was set to zero. A Poisson's ratio of zero was chosen as it would have no effect on the experimental results as lateral stresses were not considered. The sides of the chamber, which were impermeable, were modelled as such, although some lateral flow likely occurred. This approach is consistent with that used by other authors conducting confined compression experiments in connective tissues, such as in the assessment of articular cartilage [29], collagen hydrogels [30] and the intervertebral disc [23]. The material defined has been used to represent the solid matrix of articular cartilage [29] and intervertebral disc [31]. The coupled hyperelastic strain energy for this material is given by:

$$W(I_1 I_2 J) = \frac{1}{2}c\left(e^{Q} - 1\right)$$

And

$$Q = \frac{\beta}{\lambda + 2\mu}[(2\mu - \lambda)(I_1 - 3) + \lambda(I_2 - 3) - (\lambda + 2\mu)ln\,J^2$$

where I_1 and I_2 are the first and second invariants of the right Cauchy–Green tensor, J is the Jacobian of the deformation gradient and β is the exponential stiffening coefficient. λ and μ are the Lamé parameters, related to the Young's modulus (E) and Poisson's ratio (ν) as follows:

$$\lambda = \frac{E}{(1 + \nu)(1 - 2\nu)}$$

$$\mu = \frac{E}{2(1 + \nu)}$$

The relaxation function (G) was assumed to be given by:

$$G(t) = 1 + \gamma \exp\left(-\frac{t}{\tau}\right)$$

where γ is the viscoelastic coefficient and τ is the relaxation time in seconds. Strain dependent permeability was described by the following function:

$$k(J) = k_0 e^{\frac{1}{2}M(J^2 - 1)}$$

where J is the Jacobian of deformation, k_0 is the isotropic hydraulic permeability in the reference state and M is the exponential strain dependent coefficient.

The solid stress on the top surface was output and matched to experimental data via a Matlab function, which was written to iteratively reverse engineer appropriate parameters for this model for each relaxation curve using a Nelder–Mead function. Hence the stress relaxation curve for each experiment was used to iteratively determine a best fit line based on mechanical parameters of the poroviscoelastic model, identifying the mechanical parameters which best described the behaviour of the tissue in each given experiment. The exponential strain dependent coefficient and exponential stiffening coefficient were restricted from becoming negative, whilst the power law exponent (α) was held at zero. Resulting best fit parameters were analysed using one way ANOVA with Tukey post-hoc analysis. Significance was set at $p \leq 0.05$. The goodness of fit in the stress relaxation fit was assessed using a coefficient of determination as described by Soltz and Ateshian [32]:

$$R^2 = 1 - \frac{\sum(\sigma - \sigma_{est})^2}{\sum(\sigma - \bar{\sigma})^2}$$

where σ is the observed stress, σ_{est} is the estimated variable from the model and $\bar{\sigma}$ is the mean value of σ, summed over all samples time steps.

3. Results

Ten samples tested in each solution, with five from derived from the medial meniscus and five from the lateral meniscus, such that a single medial and lateral meniscus was tested in each solution. Mean sample thickness was 5.19 +/− 0.18 mm (s.d.). Mean stress relaxation curves are shown in Fig. 2. Table 1 shows mean values for the parameters derived for each sample. There was a significant difference ($p < 0.05$) in the Young's modulus, zero strain dependent permeability and viscoelastic coefficient between samples tested in deionised water/0.14 M PBS and those tested in 3 M PBS. The coefficients of determination in deionised water, 0.14 M PBS and 3 M PBS were 0.98, 0.97 and 0.75 respectively. Based on the relative values of the Young's modulus in 3 M PBS compared to 0.14 M PBS, approximately 79% of the Young's modulus of bovine meniscus is attributable to ionic effects.

4. Discussion

Ionic effects play a significant role in the mechanical stiffness of meniscal tissue. Meniscal tissue is stiffest in 0.14 M PBS, the solution which most closely represents the physiological environment. In 3 M PBS, the elimination of all ionic gradients leads to generation of a larger stress than observed in other solutions. Our results show M or β to be close to zero in all solutions – this likely reflects the small magnitude of strain tested, which suggests the tissue's stiffness is likely constant in the region tested. The viscoelastic coefficient (γ) was also found to be close to zero in 3 M PBS rendering the value of τ irrelevant. Notably, our results show that tissue permeability is higher in deionised water, where the effect of mobile ions is negated, than in 0.14 M PBS. The reasons for this are unclear and likely reflect an alteration in the complex interrelationship between the viscoelastic and poroelastic effects within the tissue [33].

The coefficient of determination show an excellent fit for data generated in deionised water and 0.14 M. The 3 M data fits the model less well. Biphasic theory assumes tissue to be comprised of two phases – a viscoelastic, porous solid phase and a liquid phase which flows through the solid. If, as our work suggests, electrical charge plays a significant role in modulating the tissue's stiffness, then biphasic theory will not account for such effects. Triphasic [34] or quadriphasic [35] theory may prove a better fit for such tissues, accounting not only for the solid and liquid phases in the tissue, but also for the ionic charge within the tissue. However, the increased number of variables in such models reduces confidence in any variables generated through finite element models, as there is an increased chance of multiple best fit solutions being present.

Potential weaknesses of our work include that we obtained samples from all meniscal regions, some authors have suggested that the mechanical properties of meniscal tissue may vary dependent on the region sampled [36]. We attempted to negate any such potential effect by testing a pair of medial/lateral menisci in each solution. We were also unable to test the tissue following a hold phase to allow equilibrium to be achieved due to persistent swelling of the tissue in deionised water at times up to 4 h – with

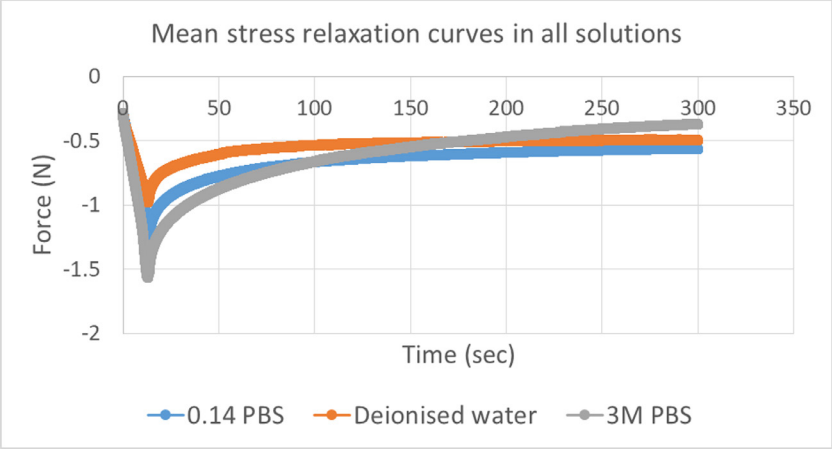

Fig. 2. Mean stress relaxation curves [standard errors: 0.14 M PBS +/−0.143 N, deionised water +/−0.098 N, 3 M PBS +/−0.1045 N).

Table 1
Comparison of derived mechanical parameters across all three solutions.

Mean values +/− s.d.						
Solution	E (Young's Modulus) (MPa)	K_0 (zero strain dependent permeability) (x10^{-15} m^4/Ns)	M (exponential strain dependent coefficient)	β (exponential stiffening coefficient)	γ (viscoelastic coefficient)	τ (relaxation time)(secs)
0.14 M PBS	0.42+/−0.32	0.53+/−0.39	0.00+/−0.00	0.11+/−0.19	0.55+/−0.26	68.85+/−24.39
Deionised water	0.38+/−0.23	0.89+/−0.59	0.00+/−0.00	0.00+/−0.00	0.59+/−0.18	52.30+/−19.78
3 M PBS	0.09*+/−0.11	0.06*+/−0.02	0.01+/−0.03	0.27+/−0.85	0.07*+/−0.00	–

*$p < 0.05$ compared with 0.14% PBS/deionised water.

longer testing times resulting in poorer fit. However, Korhonen and Jurvelin [19] suggest that in fact, the swelling of cartilage tissue in this manner leads to alteration in the pre-tension of collagen fibrils within the solid matrix, which itself may lead to a change in compressive modulus. Our experimental technique negates any such effects. We used fresh frozen tissue and although the literature suggests that freezing meniscus does not alter its structure or proteoglycan content, results obtained using fresh samples may differ from our findings – though such an approach is logistically challenging. The effect of post-mortem time on samples is unknown, though all samples were tested within 6 weeks of slaughter. The post-mortem time was similar between groups such that any confounding effect of post-mortem time would exhibit itself across all groups equally and would hence not affect experimental validity. Finally, we have not assayed our tissues for proteoglycan content and assume that these ionic effects are secondary to the effect of proteoglycans in common with other connective tissues such as cartilage and intervertebral disc.

In conclusion, we demonstrate that ionic effects significantly influence the mechanical stiffness of the bovine meniscus, contributing to approximately 79% of the Young's modulus measured in tissue plugs. Although biphasic theory has been used to describe this tissue in the literature, our work suggests that it is necessary to include the influence of ionic effects when developing mathematical models of this tissue, particularly in situations where fluid flow or localised strain is modelled.

Competing interests

None declared.

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None.

Ethical approval

Not required.

References

[1] Rai MF, McNulty AL. Meniscus beyond mechanics: Using biology to advance our understanding of meniscus injury and treatment. Connect Tissue Res 2017;58:221–4.
[2] Shrive NG, O'Connor JJ, Goodfellow JW. Load-bearing in the knee joint. Clin Orthop Relat Res 1978;131:279–87.
[3] Shrive N. The weight bearing role of the menisci of the knee. J Bone Jt Surgery. Br Vol 1974;56B:381.
[4] Seedhom BB, Dowson D, Wright V. Functions of the menisci - a preliminary study. J Bone Jt Surgery 1974;56B:381–2.
[5] Fithian DC, Kelly MA, Mow VC. Material properties and structure-function relationships in the menisci. Clin Orthop Relat Res 1990;252:19–31.
[6] McDermott ID, Masouros SD, Amis AA. Biomechanics of the menisci of the knee. Curr Orthop 2008;22:193–201.
[7] Furumatsu T, Kanazawa T, Yokoyama Y, Abe N, Ozaki T. Inner meniscus cells maintain higher chondrogenic phenotype compared with outer meniscus cells. Connect Tissue Res 2011;52:459–65.
[8] Halper J. Proteoglycans and Diseases of Soft Tissues. Adv. Exp. Med. Biol. 2014;802:49–58.
[9] McDevitt CA, Webber RJ. The ultrastructure and biochemistry of meniscal cartilage. Clin Orthop Relat Res 1990;252:8–18.
[10] Roughley PJ, McNicol D, Santer V, Buckwalter J. The presence of a cartilage-like proteoglycan in the adult human meniscus. Biochem J 1981;197:77–83.
[11] Mcnicol D, Roughley PJ. Extraction and characterization of proteoglycan from human meniscus. Biochem J 1980;185:705–13.
[12] Adams ME, Muir H. The glycosaminoglycans of canine menisci. Biochem J 1981;197:385–9.
[13] Nakano T, Dodd CM, Scott PG. Glycosaminoglycans and proteoglycans from different zones of the porcine knee meniscus. J Orthop Res 1997;15:213–20.
[14] Fox AJS, Wanivenhaus F, Burge AJ, Warren RF, Rodeo SA. The human meniscus: a review of anatomy, function, injury, and advances in treatment. Clin Anat 2015;28:269–87.
[15] Canal Guterl C, Hung CT, Ateshian GA. Electrostatic and non-electrostatic contributions of proteoglycans to the compressive equilibrium modulus of bovine articular cartilage. J Biomech 2010;43:1343–50.
[16] Han E, Chen SS, Klisch SM, Sah RL. Contribution of proteoglycan osmotic swelling pressure to the compressive properties of articular cartilage. Biophys J 2011;101:916–24.
[17] Korhonen RK, Laasanen MS, Töyräs J, Lappalainen R, Helminen HJ, Jurvelin JS. Fibril reinforced poroelastic model predicts specifically mechanical behavior

of normal, proteoglycan depleted and collagen degraded articular cartilage. J Biomech 2003;36:1373–9.
[18] Parsons JR, Black J. Mechanical behavior of articular cartilage: quantitative changes with alteration of ionic environment. J Biomech 1979;12:765–73.
[19] Korhonen RK, Jurvelin JS. Compressive and tensile properties of articular cartilage in axial loading are modulated differently by osmotic environment. Med Eng Phys 2010;32:155–60.
[20] Räsänen LP, Tanska P, Zbýň Š, van Donkelaar CC, Trattnig S, Nieminen MT, et al. The effect of fixed charge density and cartilage swelling on mechanics of knee joint cartilage during simulated gait. J Biomech 2017;61:34–44.
[21] Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE. New in vivo measurements of pressures in the intervertebral disc in daily life. Spine (Phila Pa 1976) 1999;24:755–62.
[22] Buckwalter JA, Smith KC, Kazarien LE, Rosenberg LC, Ungar R. Articular cartilage and intervertebral disc proteoglycans differ in structure: An electron microscopic study. J Orthop Res 1989;7:146–51.
[23] Heneghan P, Riches PE. The strain-dependent osmotic pressure and stiffness of the bovine nucleus pulposus apportioned into ionic and non-ionic contributors. J Biomech 2008;41:2411–16.
[24] Gelber PE, Gonzalez G, Torres R, Garcia Giralt N, Caceres E, Monllau JC. Cryopreservation does not alter the ultrastructure of the meniscus. Knee Surg Sports Traumatol Arthrosc 2009;17:639–44.
[25] Qu C, Hirviniemi M, Tiitu V, Jurvelin JS, Töyräs J, Lammi MJ. Effects of freeze-thaw cycle with and without proteolysis inhibitors and cryopreservant on the biochemical and biomechanical properties of articular cartilage. Cartilage 2014;5:97–106.
[26] Mow VC, Schoonbeck JM. Contribution of Donnan osmotic pressure towards the biphasic compressive modulus of articular cartilage. Trans Orthop Res Soc 1984;9:262.
[27] Maas SA, Ellis BJ, Ateshian GA, Weiss JA. FEBio: finite elements for biomechanics. J Biomech Eng 2012;134:011005.
[28] Holmes MH, Mow VC. The nonlinear characteristics of soft gels and hydrated connective tissues in ultrafiltration. J Biomech 1990;23:1145–56.
[29] Ateshian GA, Warden WH, Kim JJ, Grelsamer RP, Mow VC. Finite deformation biphasic material properties of bovine articular cartilage from confined compression experiments. J Biomech 1997;30:1157–64.
[30] Busby GA, Grant MH, MacKay SP, Riches PE. Confined compression of collagen hydrogels. J Biomech 2013;46:837–40.
[31] Iatridis JC, Setton LA, Foster RJ, Rawlins BA, Weidenbaum M, Mow VC. Degeneration affects the anisotropic and nonlinear behaviors of human anulus fibrosus in compression. J Biomech 1998;31:535–44.
[32] Soltz MA, Ateshian GA. Interstitial fluid pressurization during confined compression cyclical loading of articular cartilage. J Biomech 1998;31:927–34.
[33] Hosseini SM, Wilson W, Ito K, van Donkelaar CC. How preconditioning affects the measurement of poro-viscoelastic mechanical properties in biological tissues. Biomech Model Mechanobiol 2013;13:503–13.
[34] Lai WM, Hou JS, Mow VC. A triphasic theory for the swelling and deformation behaviors of articular cartilage. J Biomech Eng 1991;113:245–58.
[35] Huyghe JM, Janssen J. Quadriphasic mechanics of swelling incompressible porous media. Int J Eng Sci 1997;35:793–802.
[36] Proctor CS, Schmidt MB, Whipple RR, Kelly MA, Mow VC. Material properties of the normal medial bovine meniscus. J Orthop Res 1989;7:771–82.