Proteoglycans exert a significant effect on human meniscal stiffness through ionic effects

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Abstract

Background

Proteoglycans contribute to mechanical stiffness in articular cartilage, aiding load transmission. The magnitude of the ionic contribution of proteoglycans to the stiffness of human meniscal tissue has not been established.

Methods

Thirty-six discs of human meniscal tissue were placed within a custom confined compression chamber and bathed in three solutions of increasing ionic concentration. Following a 0.3N preload, at equilibrium, a 10% ramp compressive strain was followed by a 7200 second hold phase. A nonlinear poroviscoelastic model with strain dependent permeability was fitted to resultant stress relaxation curves. All samples were assayed for proteoglycan content. Model parameters were analysed using multivariate analysis of variance whilst proteoglycan content was compared using a univariate analysis of variance model.

Findings

A significant difference ($p<0.05$) was observed in the value of the Young's modulus (E) between samples tested in deionised water compared to those tested in solutions of high ionic concentration. No differences were observed in the zero-strain permeability or the exponential strain dependent stiffening coefficient. Proteoglycan content was not found to differ with solution; but was found to be significantly increased in the middle meniscal region of both menisci.

Interpretation

Proteoglycans make a significant ionic contribution to mechanical stiffness of the meniscus, increasing it by 58% in the physiological condition. It is therefore critical that meniscal regeneration strategies attempt to recreate the function of proteoglycans to ensure normal meniscal function.

Keywords: meniscus; proteoglycans; tissue mechanics

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1. Introduction

Whilst, historically, the orthopaedic community has been oblivious to the importance of the menisci to the normal functioning of the knee, it is now appreciated that the meniscus serves a number of functions including load transmission [1], aiding congruity of the joint surfaces [2] and in stability of the knee [3], especially in ACL (anterior cruciate ligament) deficient states [4].

It is well established [1] that the structure of the meniscus aids it in transmitting load. The electrolyte content of the meniscus is estimated at 74% [5], with 80% of the remaining dry weight being Type 1 collagen [6]. Collagen fibres are oriented circumferentially in the deep layers of the meniscus, parallel to the meniscal border, with radial and axial oriented tie fibres branching from the peripheral border of the meniscus to its inner rim, surrounding the aforementioned circumferential fibres [1,7]. The menisci are firmly anchored to the tibial surfaces at their roots. Under load, the firm attachment of the menisci at their roots prevent extrusion and allow generation of circumferential tensile hoop stresses in the circumferential collagen fibres, aiding load distribution [8]. Some superficial fibres are oriented radially, interweaving between the circumferential fibres, providing structural integrity.

As well as collagen, the meniscus is also comprised of fibrochondrocytes and proteoglycans. Proteoglycans are proteins with numerous sulphated glycosaminoglycan side chains carrying a strong negative charge. Aggrecan is the major type of large proteoglycan found within the meniscus [9] and proteoglycans are thought to comprise 2-3% of the dry weight of the meniscus [6], with the distribution of proteoglycans in the tissue varying in both frontal and coronal planes [10]. These proteins are highly hydrophilic and allow water to be trapped within the tissue, supporting the tissue under compressive load [11]. Whilst aggrecan is also present as one of the dominant proteoglycans in both articular cartilage and the nucleus pulposus of the intervertebral disc, the proportion of proteoglycans in both these tissue is close to an order of magnitude higher than that observed in the meniscus [12].

In articular cartilage, proteoglycans are understood to play a major role in maintaining the compressive stiffness of the tissue, with digestion of proteoglycans resulting in a marked reduction in its compressive modulus [13] and correlation evident between proteoglycan content and this modulus. In cartilage, the strong negative charge of proteoglycan molecules exerts a Donnan osmotic pressure: as the negatively charged moieties attached to the proteoglycans are fixed in the meniscal ultrastructure, charge is distributed unevenly across the cartilage membrane, leading to development of an electrical potential (the Donnan potential) across the cellular boundary. This, in turn leads to generation of an osmotic pressure and inflow water into the cartilage, ultimately allowing fluid to be absorbed into the cartilage to aid load resistance. Study of articular cartilage to investigate the contribution of the ionic effect of proteoglycans to stiffness of the tissue suggests that 62% of the compressive modulus at equilibrium is attributable to such effects [14]. Similar work [15] in the nucleus pulposus of the intervertebral disc suggests that 70% of the stress response is attributable to ionic effects mediated by proteoglycans, despite the population of proteoglycans in the nucleus pulposus being composed of short length monomers as opposed to the larger chain polymeric molecules observed in other tissues [16].

There has been limited exploration of the role of proteoglycans in maintaining mechanical stiffness of the meniscus to date. Evaluation of cervine meniscal stiffness using microindentation techniques following use of hyaluronidase to digest glycosaminoglycans showed a reduction of 15% in creep stiffness [17]. Also, a recent study [18] suggested that proteoglycans within the meniscus exert a significant effect on mechanical stiffness via ionic effects, although the number of samples and the

use of bovine meniscus limits how far these results can be extrapolated. Therefore, this study aims to quantify the contribution that proteoglycans make to the stiffness of the human meniscus.

2. Methods

Following ethical approval, 12 paired, fresh frozen human menisci were obtained from a tissue repository. Donors were less than 65 years of age, with no history of knee surgery, knee osteoarthritis or significant knee injury. Samples were defrosted on the morning of experimentation.

A hollow punch was used to obtain 36, 5mm diameter sections of meniscal tissue from either the anterior, middle or posterior regions of each meniscus (Figure 1). All sampling was conducted from the periphery of the meniscus to allow a cylindrical sample of sufficient dimensions to be obtained.

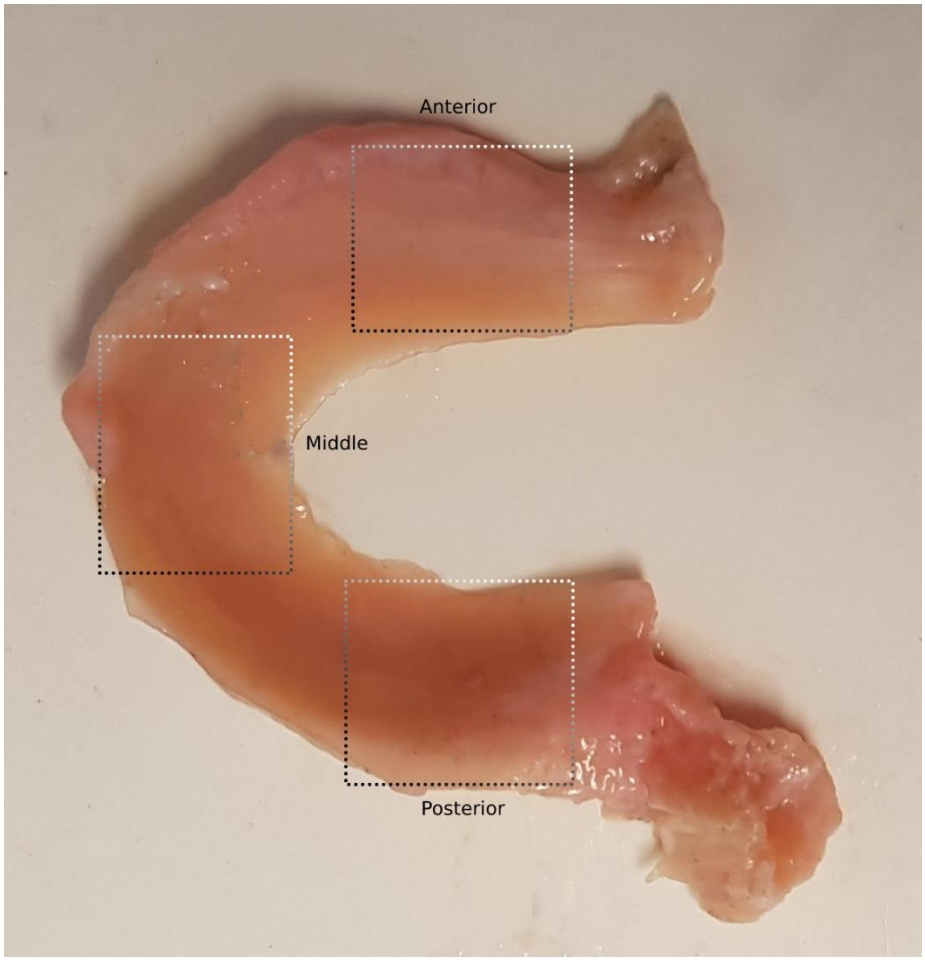

Figure 1 – Superior view of a right knee lateral meniscus illustrating meniscal sample locations

Due to the topography of the menisci, the superior and inferior surfaces of the sections were not parallel and therefore a custom-made device, which held two microtome blades precisely 2mm apart, was used to obtain a cylindrical sample from the centre of each section, such that both contact surfaces were removed. Sample thickness was determined using a micrometer screw gauge.

A bespoke confined-compression chamber, with an inner-diameter of 5mm and a bottom lattice-work of 400 μm square pores, was 3D printed. A hollow indenter, which also had a permeable lattice at its inferior aspect fitted the chamber, ensuring a small side-clearance. The indenter gripped by a BOSE 3100 materials testing machine fitted with a 22N load cell (Figure 2). Similar techniques have

been used extensively to characterise the mechanical properties of tissue such as meniscus [19] and articular cartilage [20].

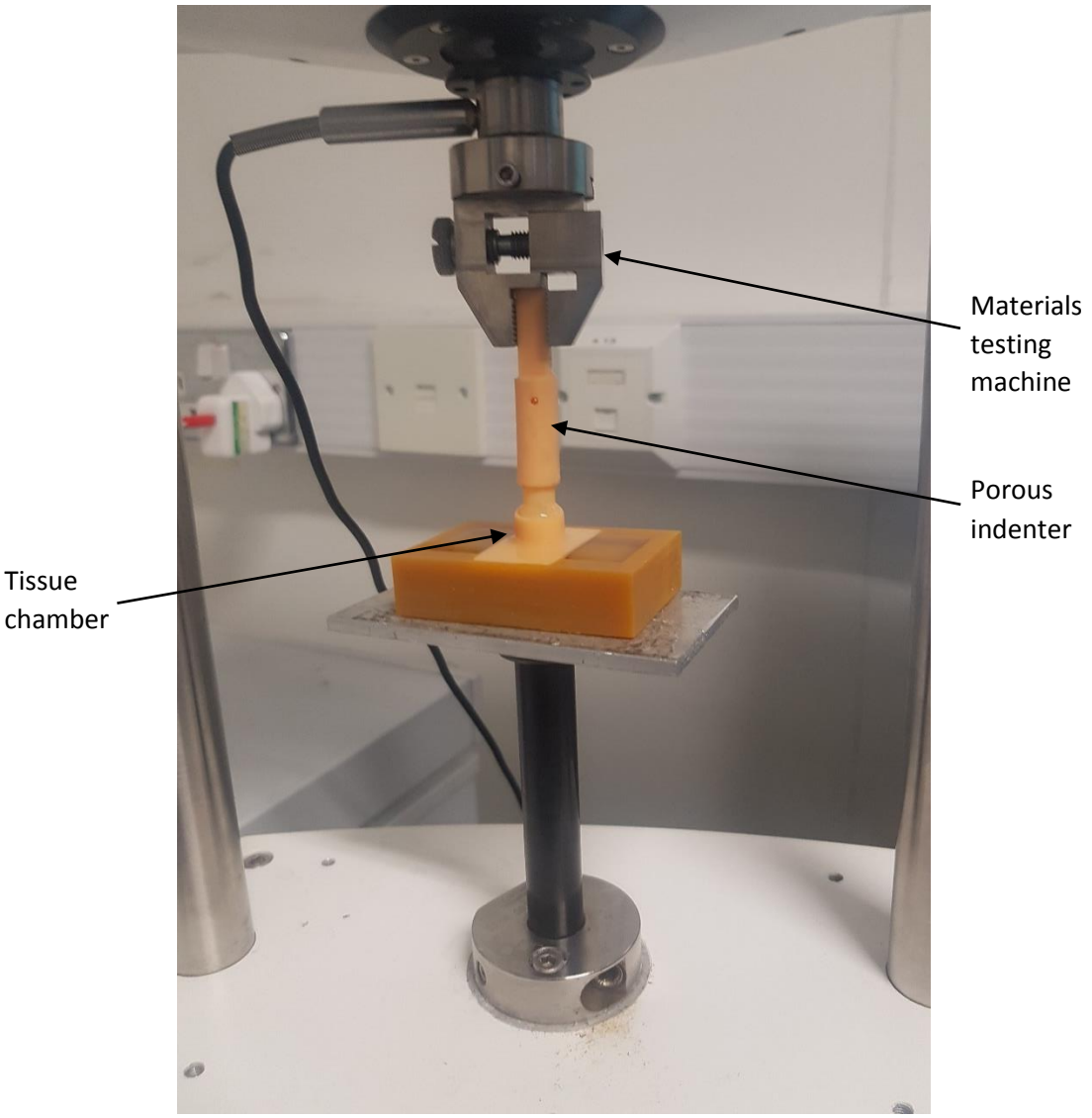

Figure 2 - Confined compression apparatus (bathing chamber removed for clarity)

Each of the 36 samples were placed within the compression chamber and bathed in in one of three solutions, such that the fluid permeated the tissue, the confining chamber and the indenter. As in previous studies [9, 14], deionised water was used to negate the osmotic effects of mobile ions within the tissue; 0.14M PBS (phosphate buffered saline) was used to mimic a physiological environment and 3M PBS negate all ionic effects. Four samples from the anterior, middle and posterior regions were tested in each solution, such that specimens from different anatomical regions were distributed evenly across each experimental group.

The meniscal surface was identified by lowering the indenter until a load of 0.3N was registered. The sample was then allowed to equilibrate, holding the displacement of the indenter constant. The time to equilibrium was determined by holding the displacement until the force measured did not vary by >2% over a 30 minute period. Time to reach equilibrium was 2 hours for samples tested in 0.14M PBS and 3M PBS, whilst samples tested in deionised water were left to equilibrate for 15 hours. A

10% compressive strain was applied, at 1% per second, before a hold-phase at constant strain of 7200 s. Each sample was tested once.

A finite element model was constructed using FEBio software using a non-linear, large strain, biphasic, poroviscoelastic model with strain dependent permeability. This model assumes the tissue as having two phases. The viscoelastic solid phase was defined by an elastic stress given by (Homes and Mow, 1990)

$$\sigma_e = \frac{1}{2} H_M \left(\frac{\lambda^2 - 1}{\lambda^{2\beta+1}} \right) e^{\beta(\lambda^2-1)}$$

where λ is the stretch ($\lambda = 1 + \varepsilon$); H_M the gradient of the stress-strain curve at $\lambda = 1$; and β a stiffening coefficient associated with the sensitivity of σ_e to large strain. The solid phase's relaxation function $G(t)$ was described by

$$G(t) = 1 + G_1 e^{-\frac{t}{\tau_1}}$$

The fluid phase was an incompressible Newtonian fluid. Time-dependent behaviour is associated with the resistance to the flow of fluid through the solid via Darcy's law and characterised by the hydraulic permeability (permeability divided by the viscosity of the permeating fluid). A variation on the Holmes-Mow model of strain-dependent permeability was used, i.e.

$$k(\lambda) = k_0 \left(\frac{\lambda - \phi_0}{1 - \phi_0} \right)^{\alpha} e^{[M(\lambda^2-1)/2]}$$

where the hydraulic permeability is dependent on the stretch and ϕ_0 is the zero-strain porosity. The zero-strain permeability, k_0, and the exponential strain dependent coefficient, M, were restricted from becoming negative, whilst the power law exponent, α, was held at zero, reducing the above equation to

$$k(\lambda) = k_0 e^{[M(\lambda^2-1)/2]}$$

Such a model has been used in the literature to describe both articular cartilage and meniscus [19,21]. The model consisted of 404 nodes, with 100 elements. A convergence study was conducted to investigate an appropriate model size, this suggested a percentage error of <0.1% for a model with 404 nodes compared to one with ~1000 nodes. Hence a 404-node model was chosen to allow an acceptable compromise between accuracy and computational calculation efficiency. Boundary conditions were set appropriate to confined compression, and the Poisson's ratio was set to zero, so that for small strains, $H_M \cong E$, the Young's modulus.

Model parameters were determined by fitting the experimental force on the sample in the relaxation phase to the calculated force in the FE model using the fminsearch function in Matlab (Mathworks, Massachusetts, USA) and E, k_0, M, β, G_1 and τ_1 as fitting parameters. After each iteration, matlab amended the model and called the FEBio solver. The goodness of fit in the stress relaxation fit was assessed using a coefficient of determination as described by Soltz and Ateshian [19]. Converged best fit parameters were compared using multivariate ANOVA with Bonferroni correction for multiple comparisons, with meniscal side, meniscal region and bath osmolarity as fixed factors and with significance set at $p \leq 0.05$. Once tested, samples were immediately re-frozen.

Proteoglycan content in each sample was determined by a proprietary assay (Biocolour Ltd, County Antrim, UK). Prior to applying the assay, all samples were washed with deionised water 10 times to remove any excess salt, as the results of the assay could be skewed by presence of excessive salt.

A papain extraction reagent was prepared by adding 800mg sodium acetate, 400mg EDTA and 40 mg cysteine hydrochloride to 100ml of a 0.2M sodium phosphate buffer. The pH of this solution was corrected to 6.4 and 250 microlitres of a papain crystallised suspension was added. Each sample was then cut in half using a scalpel and its wet weight was recorded – the assay required a wet weight of 20-50mg. Individual samples were them placed in labelled microcentrifuge tubes with 1ml of the papain extraction reagent. All samples were placed in a warm water bath (65°C) and set to shake slowly. Samples were left overnight to digest, then centrifuged at 10000g for 10 minutes

50µl of the supernatant of each test sample was added to individual microcentrifuge tubes and made up to 100µl using the previously prepared papain extraction reagent. Tubes containing 1,2,3,4,5µg of the assay reference standard were also prepared and made up to 100µl in a similar fashion. A millilitre of the assay dye reagent was then added to each sample.

All samples were placed on a mechanical shaker for 30 minutes, during which time a precipitant was observed to form, a further 10 minutes of centrifugation was undertaken at 12000g. The supernatant from each tube was then carefully removed and 0.5ml of the dye dissociation reagent added. A vortex mixer was used to allow the bound dye to dissolve into solution and 200µl of each sample was transferred to a 96 microwell plate. A microplate reader was used to measure absorbance at 656nm.

The luminescence values for the assay reference standard solutions were used to create a standard curve. A best fit line was applied to this curve using Microsoft Excel (Microsoft, Redmond, Washington, United States) – the equation for this line was then used to calculate proteoglycan content for individual samples. Proteoglycan content was compared between samples using univariate ANOVA, with significance set at $p \leq 0.05$. Proteoglycan content was considered as the sole dependent variable, with the solution tested, the meniscus tested, and the region of the sample considered as fixed factors.

3. Results

Thirty six samples were obtained from 12 menisci, three from each meniscus. Mean sample thickness was 1.99 (SD 0.04) mm.

Following best fitting, the viscoelastic coefficient, G_1, and relaxation time, τ_1, were close to zero in all samples, hence finite element modelling was conducted twice for all samples: with and without $G_1 = 0$. No difference was observed in the other material parameters between these two conditions, and thus it was assumed that no viscoelastic behaviour of the solid phase occurred. If one assumes that solid phase viscoelasticity is related to the viscoelastic stress relaxation of collagen fibre tension in the matrix, compression and buckling of the fibrous solid phase could be considered unlikely to elicit a viscoelastic effect. G_1 was therefore prescribed to be zero and is not presented or discussed further.

Table 1 shows the derived values for the mechanical parameters of the tissue in each solution.

Parameter mean values (95% confidence interval)				
Solution	**E (Young's Modulus) (MPa)**	**k_0 (zero strain dependent permeability) ($x10^{-16}m^4/Ns$)**	**M (exponential strain dependent coefficient)**	**β (exponential stiffening coefficient)**
Deionised water	1.15 (0.94-1.35)*	0.08 (0.00-0.22)	0.01 (0.10-0.12)	0.23 (0.18-0.28)
0.14M PBS	0.68 (0.48-0.89)	0.24 (0.10-0.38)	0.01 (0.10-0.12)	0.23 (0.18-0.28)
3M PBS	0.43 (0.22-0.63)	0.18 (0.04-0.32)	0.01 (0.10-0.12)	0.21 (0.17-0.26)
* p<0.05 compared to 0.14M/3M PBS.				

Table 1 – Parameter mean values

A significant difference was observed in the value of the Young's modulus between samples tested in deionised water compared to those tested in either 0.14M ($p = 0.01$) or 3M PBS ($p < 0.01$). No significant differences were observed in either the value of the zero-strain hydraulic permeability or the exponential strain dependent, or the exponential stiffening coefficient between solutions. Furthermore, no significant differences were observed in the value of any of the mechanical parameters when comparing meniscal side or meniscal region. The mean R^2 values, assessing goodness of fit, were 0.83, 0.75 and 0.76 in deionised water, 0.14M PBS and 3M PBS respectively, with an overall mean R^2 of 0.78 +/- 0.11 (s.d.).

Proteoglycan content was not found to differ significantly with solution: Table 2 illustrates the mean concentration of proteoglycans across solutions. However, proteoglycan content was found to be increased in the middle region of each meniscus ($p=0.043$ vs anterior samples, $p = 0.036$ vs posterior samples).

Region	**Proteoglycan content (µg/ g of tissue) [95% confidence interval]**
Deionised water	196.05 [159.37-232.73]
0.14M PBS	151.28 [114.60-187.96]
3M PBS	148.67 [139.46 – 212.82]

Table 2 - Proteoglycan content

4. Discussion

Proteoglycans make a significant ionic contribution to mechanical stiffness of the human meniscus, in the non-diseased state, increasing the meniscal stiffness by 58% in the physiological condition compared to the 3M state. Despite the marked difference in the concentration of proteoglycans in meniscus compared to articular cartilage or intervertebral disc, the magnitude of this contribution is

not too dissimilar to that described for these tissues. Hence, proteoglycans are integral to meniscal function and any efforts to repair or re-constitute the tissue should account for their function.

The ramifications of this finding could be clinically significant. To our knowledge, there have been no studies to date exploring the constitution of either healed meniscal tissue following primary repair or allograft tissue following transplantation with respect to its proteoglycan content and whether this is similar to that observed in the native tissue. An animal study has suggested that supplementation of meniscal repairs with hepatocyte growth factor/ platelet derived growth factor results in increased proteoglycan staining compared to that observed in menisci repaired without growth factors [22]. Furthermore, evaluation of biopsies of the Actifit meniscal scaffold have shown proteoglycans in only a proportion of samples, albeit in a small patient population [23]. Interestingly, seeding such scaffolds with biologically active constituents such as growth factors or stem cells may provide a means through which to encourage healing and/or proteoglycan reconstitution, an option which is being explored [24]. If meniscal defects heal without the presence of proteoglycans within the tissue, the resultant tissue is likely to be less stiff than native meniscus and may therefore be inferior in its ability to transmit load, leading to abnormal stresses on adjacent articular cartilage.

The tissue is markedly stiffer in deionised water than in either PBS solution. This can be explained by the fact that, in the absence of mobile ions, the stiffness of the tissue is significantly augmented by the osmotic pressure generated by the difference in fixed charge density between the internal and external environment. Circulating mobile ions internally and externally in the physiological condition reduce the electrostatic disparity, whilst the hypertonic condition is designed to nullify the electrostatic effects completely. Although it did not reach significance, the permeability of the tissue was also lowest in deionised water. Transiently, an isotonic permeate retains both mobile and fixed ion gradients which enhance fluid flow within tissue for a given mechanical fluid pressure [25]. Thus, the lower permeability seen in hypertonic and hypotonic solutions are not mechanical low permeability pe se, rather reduced, apparent permeabilities due to the loss of these gradients. Nonetheless, whilst a biphasic model can identify and partition the overall effects of the osmolarity of the permeate, a triphasic [26] or quadriphasic model [27] is required to truly and fully describe the physics of the experiment.

Neither the exponential stiffening nor strain dependent coefficients differed between solutions. These variables seem unaffected by ionic changes within the solution; however, their similarity adds credence to the reliability of the experimental technique.

Whilst we fitted a nonlinear poroviscoelastic model to the data, G_1 and τ_1 were found to be zero, indicating one of two things: either the collagen network did not exhibit stress relaxation, or that the curve fitting algorithm iterated towards a local best-fit solution in which G_1 and τ_1 were equal to zero. In confined compression, the likelihood of stress relaxation within collagen fibres themselves is difficult to argue and thus we suggest this finding infers this. Regardless, this model does not consider ionic effects and future work may explore the appropriateness of a triphasic model, described by Lai et al [26], in predicting the behaviour of meniscal tissue. Similar to a poroviscoelastic model, such an approach is challenging due to the likelihood of obtaining multiple 'false positive' solutions [28] due to the multitude of parameters being fitted.

Reassuringly, there were no differences in proteoglycan content between solutions – a potential confounder for our experiments. Nevertheless, we found that proteoglycan content is increased in the middle region of each meniscus. Interestingly, this did not lead to an increase in stiffness of this region compared to anterior or posterior samples, suggesting that the maximal ionic contribution of

proteoglycans to meniscal stiffness is either not concentration dependent, or limited by other factors.

Our study has a number of strengths. We have tested a large number of samples, using a common technique. By testing samples at equilibrium, we have excluded any potential effects of swelling, which can be significant in meniscal tissue [29]. Potential weaknesses of our work are the use of frozen tissue, although fresh frozen tissue is commonly used in biomechanical testing. Nonetheless, examination of fresh tissue may show different results. We were unaware of the post-mortem time for any of our samples. As well as this, we did not test samples selectively from a single meniscal region, but rather chose to test equal numbers of samples from each region, as this would have required a large number of menisci. Previous work [30] has suggested that mechanical behaviour of meniscal tissue is altered dependent on the region it is derived from, our work did not show such a difference, although we did have small numbers of samples from each region. In any case, testing equal numbers of samples from each meniscal region allowed for any effect of such differences to remain equal between solutions. As well as this, our samples were all derived from the periphery of the meniscus – proteoglycan concentration has been shown to be highest in the inner zones of the meniscus [31]. Our sampling technique did not allow us to differentiate between the vascular zones of the meniscus. We also did not use protease inhibitors to prevent specimen degradation.

A mean R2 value of 0.78 +/- 0.11 suggest good, but not excellent fits, with the model struggling to fit at early times in the hold phase. The equilibrium force determined Young's modulus and thus the permeability value controlled not only the initial peak compressive force, but also the rate of stress relaxation observed. In decreasing k0 one obtains a higher negative peak force, but slower mechanics, whilst increasing k0, decreased peak force and increased the rate of relaxation. It was difficult for the model to accurately capture both the peak load and the rate of relaxation, and in the majority of cases a compromise k0 was converged to. Additional model parameters (beta, G1 tau1 etc.) were not able to further improve the fit. Thus, the constitutive behaviour of the model and its appropriateness for meniscal tissue warrants additional research.

Whilst the ionic concentrations sampled here are supra-physiological, this works highlights that proteoglycans significantly contribute to meniscal stiffness at physiological ion concentrations via ionic effects. Whilst no prosthetic replacement for meniscal tissue currently exists, there has been interest in the use of meniscal scaffolds such as the Actifit (Orteq Ltd, Wimbledon, London, United Kingdom) and Collagen Meniscus Implant (Ivy Sports Medicine GmbH, Grafelfing, Germany). Tissue ingrowth following the use of such implants has been found to be composed of collagen [32,33], however, there has been no consideration of whether proteoglycans are adequately restored. This work highlights the importance of restoring normal proteoglycan function is such meniscal preservation strategies.

Conclusion

Proteoglycans make a significant contribution via ionic effects to the stiffness of the human meniscus, increasing the stiffness by 58%. These data suggest that meniscal preservation strategies should take this contribution into account and seek to reconstitute proteoglycans within the tissue, allowing repaired tissue to mimic the properties of the native tissue.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: