Biaxial testing of canine annulus fibrosus tissue
under changing salt concentrations

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1 Abstract

The in vivo mechanics of the annulus fibrosus of the intervertebral disc is one of biaxial loading rather than uniaxial loading. The material properties of the annulus are intimately linked to the osmolarity in the tissue. This paper presents biaxial relaxation experiments of canine annulus fibrosus tissue under stepwise changes of external salt concentration. The force tracings show strong time, salt concentration and orientation dependent stresses. The force tracing signature of a response to a change in strain, is one of a jump in stress that relaxes partly as the new strain is maintained. The force tracing signature of a step wise change in salt concentration is a progressive monotonous change in stress towards a new equilibrium value. Though the number of samples does not allow any definitive quantitative conclusions, the trends may shed light on the complex interaction between directionality of forces, strains and fiber orientation on the one hand and on the osmolarity of the tissue on the otherhand. The dual response to a change in strain is understood as an immediate response before fluid flows in or out of the tissue, followed by a progressive readjustment of the fluid content in time because of the gradient in fluid chemical potential between the tissue and the surrounding solution.

Keywords: swelling, collagen, osmosis, Donnan, cartilaginous.
2 Introduction

Intervertebral disc tissue is a very deformable molecular mixture of an ionized solid and an ionised fluid. It is composed of a gelly-like core, the nucleus pulposus, enclosed within a fibrous ring, the annulus fibrosus. Both are composed of a crosslinked collagen network embedded into a gel of hydrated proteoglycans, be it in different ratios. The swelling propensity of the tissue is primarily generated by the ionized proteoglycans. Though it is estimated that 20 % of the water is absorbed by the collagen network. Different modelling strategies have been proposed to grasp its coupled electro-chemo-mechanical properties (Huyghe, 1999; Iatridis et al., 2003; van Loon et al., 2003; Huyghe et al., 2003; Schroeder et al., 2006, 2008). The constitutive relationships of intervertebral disc tissue are subdivided into two categories (Huyghe and Janssen, 1997). One set relates the free energy of the mixture, the stress and the electrochemical potential to its composition and its deformation (Huyghe et al., 2003). The other relates fluxes to gradients in electrochemical potentials. All of these parameters are generally found to be dependent on orientation and position within the disc (Houben et al., 1997), position of the disc within the spine and dependent on age and species. This paper explores an experimental method to study couplings associated with canine annulus fibrosus tissue chemomechanics. We present three explorative pilot experiments each of them including relaxation, swelling and shrinking. While in an earlier paper (Huyghe and Drost, 2004), we restricted our attention to uni-axial experiments, the present paper reports on biaxial experiments only.
3 Methods

Lower lumbar motion segments were cut from spines of two mongrel dogs and were stored at -65 degrees centigrade. One of the vertebral bodies of a motion segments was sawed away almost completely, so that at most a thin layer of bone remained on top of the intervertebral discs. The posterior half of the segment (arch) was sawed away. The anterior part of the motion segment was allowed to thaw. The surrounding muscle and fat tissues were removed with a scalpel. The nucleus pulposus was carefully removed. During this process the disc was kept moist by dripping with a physiological salt solution. Lamellae at the anterior part of the annulus fibrosus and of at least 10 mm length and a minimal number of branches were selected under the stereo microscope. Lamellae were separated by blunt dissection. Finally a sample of circumferentially 10 mm by axially 2 to 8 mm is cut. The thickness of the lamellae was inhomogenous and variable and was about 1 mm. The anterior side of each sample was glued upon a flexible polystyrene frame of 15 to 18 mm (Fig. 1) using cyanoacrylate glue (Hyloglue M-100). The frame was then cut diagonally to create four lips at the four sides of the sample, two of 15 mm width and two of 18 mm width. Traction exerted on two opposite 15 mm lips resulted in extension of the samples along their long axis, corresponding to the circumferential direction in the disc while traction exerted on two opposite 18 mm lips resulted in extension of the samples along their short axis, corresponding to traction in the axial direction in the disc. The long axis of the sample had an angle of approximately 30 degrees with the collagen fiber direction. The thickness of the polystyrene lips was 0.5 mm. The 3 samples (Table 1) were stored at -65 degrees centigrade until use.
A sample was taken out of the freezer, allowed to thaw and after about 15 minutes mounted into a horizontal mechanical drawing bench by clamping the polystyrene lips. The 15 mm lips were gripped in two opposite clamps moving in the x-direction and the 18 mm lips were gripped in two opposite clamps moving in the y-direction. The procedure is based upon the work of Myers et al. (1984) and Akizuki et al. (1986) and Huyghe and Drost (2004). The displacement of two x-clamps of the drawing bench were computer controlled. The tensile forces in x and y directions were measured by means of two force transducers. Above the samples two dripping devices were mounted, one filled with distilled water, and one filled with a physiological salt solution. The external salt concentration and the clamp position were changed in a stepwise fashion.

4 Results

The force tracings associated with three different samples are shown in Figs. 2-5. The experimental measurement on the first sample is reported in two different figures (Fig. 2 and 3) reporting measurement of force in intervals of time separated by half an hour rest. The thickness of sample 1 is less than 1 mm. The width is about 8 mm. A -12 % step in the y-strain is applied at t=0 and a +6 % step at t=10 min. A +3 % step in the x-strain is applied at t=15 min. The external salt concentration is physiological except between t=20 min and t=25 min where it is zero. The sample is taken halfway the outer edge of the annulus fibrosus and the nucleus-annulus interface. The clamp speed at which the step in the strain is applied is 0.05 mm/s. This is sufficiently fast compared to the time constant of the observed phenomena and sufficiently slow
to avoid inertia of the device to come into play. The step in y-strain results in a sharp increase in y-force and a much weaker response of the x-force. The step in the x-strain results in a sharp increase of x-force and a much weaker response of y-force. The responses to salt concentration changes are mild in sample 1. Contact with distilled water results in increase of the forces in absolute values (fig.3). The experimental measurement of forces on sample 2 are shown in Fig. 4, while those associated with sample 3 are shown in Fig. 5. The specifics of the applied boundary conditions are found in the respective legends of the figures. In Fig. 4, the x-force has a minor response to changes in salt concentration and a mayor response to the imposed steps of x-strain, while the y-force does the opposite. The y-force responds to a decrease in salt concentration from physiological to zero by a forceful move towards compressive scales. All of the samples exhibit a creep behaviour which continues long after changes in external bath concentrations and strain.

5 Discussion

The force tracings show strong time, salt concentration and orientation dependent stresses. The force tracing signature of a response to a change in strain, is one of a jump in stress that relaxes partly as the new strain is maintained. The force tracing signature of a step wise change in salt concentration is a progressive monotonous change in stress towards a new equilibrium value. When the external concentration $c_{ext}$ of NaCl is reduced from 0.15 M to 0 M, the result is an immediate decrease in external osmotic pressure $\pi = 2RTc_{ext}$ and hence a steep gradient of the water chemical potential and electrochemical potentials of
the ions between the tissue and the solution. These gradients induce an influx of water into the tissue and an outflux of ions from the tissue. Volumewise the outflux of ions is negligible compared to the influx of water. The influx of water results in a change in clamp force with time. The time constant associated with the change in salt concentration is typically longer than the time constant associated with the step changes in strain. The response to the changes in strain represent a minimal adjustment of the ionic concentrations and a major redistribution of the fluid between the sample and the external salt solution. The response to a change in salt concentration represent a major redistribution of both the salt and the fluid. The time constant $\Delta t$ associated with this fluid movement is typically

$$\Delta t = \frac{(\Delta z)^2}{K H}$$

while the time constant for the redistribution of the salt is

$$\Delta t = \frac{(\Delta z)^2}{D}$$

if we assume convection is negligible. In eqs. (1-2) $K$ is the hydraulic permeability, $H$ the transverse aggregate modulus of the sample and $D$ the diffusion coefficient of the salt and $\Delta z$ is the thickness of the sample. The results seem to indicate that the redistribution of the salt takes more time than the fluid redistribution:

$$KH \geq D$$

Even if $KH$ is in the same order as $D$, convection should slow down a response to a salt concentration, because the salt and the water typically run in opposite directions following a change in salt concentration. Whether a difference
of diffusion speed, or convection is the reason for the slower response to the salt concentration change is dilemma to be addressed in future research. The change in strain in one direction is usually accompanied by changes in stress in both directions. Similarly, a change in salt concentration results in readjustment of stress in both directions, be it in different measures. Though the small number of samples does not allow any definitive quantitative conclusions, the trends shown in the tracings may shed light on the complex interaction between directionality of forces, strains and fiber orientation on the one hand and on the osmolarity of the tissue on the other hand. The dual response to a stepwise change in strain is understood as an immediate response before fluid flows in or out of the tissue, followed by a progressive readjustment of the fluid content in time because of the gradient in fluid chemical potential between the tissue and the surrounding solution. The sensitivity to changes in salt concentration can be qualitatively understood in the context of chemoelectromechanical theory (Huyghe and Janssen, 1997).

In Fig. 4, the x-force has a minor response to changes in salt concentration and a mayor response to the imposed steps of x-strain, while the y-force does the opposite. The behavior can be understood as the response of a sample in which the fibers are primarily oriented parallel to the x-axis. Indeed, the y-response is dominated by the proteoglycan gel swelling and shrinking under changing salt concentration while the x-response is sensitive to strains because of the stiffness of the fibers.

Unlike in Fig. 4, the responses in Fig. 5 of both force tracings to changes in x-strain and salt concentrations are similar. The response of the x-force to
changes in x-strain, however is more forceful than the corresponding response of
the y-force. The response of the y-force to changes in salt concentrations is more
pronounced than the corresponding response of the x-force. This result seems
to indicate a more isotropic and cross-linked distribution of fibers in the sample
3 compared to sample 2. Elastic stiffness as computed from these experiments
is rather inaccurate, because of ill-defined cross sectional areas and specimen to
specimen variability. The elastic stiffness is between 10 and 20 MPa, and is defi-
nitely higher than the stiffnesses computed from uniaxial experiments (Galanta,
1967; Huyghe and Drost, 2004), which in turn are somewhat higher than typical
values of confined compression tests (Houben et al., 1997; Huyghe et al., 2003).
The higher stiffnesses observed in tensile versus compressive experiments are
observed in articular cartilage as well (Stoltz and Ateshian, 2000).

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References


7 Legends

Figure 1
Geometry of the polystyrene frame that is used to fix the samples into the biaxial drawing bench.

Figure 2
x-force recording (discontinuous line) and y-force recording (continuous line) as a function of time in experiment a of sample 1. The thickness of the sample is less than 1 mm. The width is about 8 mm. A -12 % step in the y-strain is applied at t=0 and a +6 % step at t=10 min. A +3 % step in the x-strain is applied at t=15 min. The external salt concentration is physiological except between t=20 min and t=25 min where it is zero. The sample is taken halfway the outer edge of the annulus fibrosus and the nucleus-annulus interface. The clamp speed at which the step in the strain is applied is 0.05 mm/s.

Figure 3
x-force recording (discontinuous line) and y-force recording (continuous line) as a function of time in experiment b of sample 1. This experiment is the continuation of experiment a of sample 1 after a 30 min interruption of the force measurement. During the break the clamp positions in both x and y directions have been untouched. At t=0 min a stepwise increase in x-strain of 6 % is applied. The y-clamps are immobile during the whole experiment. At t=60 min, a stepwise increase in x-strain of 6 % is applied again. The external salt concentration is physiological except during the periods between t=20 min and t=40 min and between 80 min and 100 min in which it is zero. The clamp speed at which the step in the strain is applied is 0.05 mm/s.
Figure 4

Clamp force recordings in x-direction (black) and clamp force recording in y-direction (blue) as a function of time in experiment 2. A solid black dot along the force scale indicates the zero force level for the x-tracing. A solid blue dot along the force scale indicates the zero force level for the y-trace. A 3 % step in the strain is applied at t=205 min and an additional 6 % step in the strain at t= 375 min in the x-direction. The strain in the y-direction is constant throughout the experiment. The external salt concentration is physiological except in the periods between t=50 min and t=145 min, between t=255 min and t=330 min and in the period t=400 min and 430 min in which the external salt concentration is zero. The thickness of the sample is about 1 \textit{mm}. The sample is taken from the outer annulus region. The clamp speed at which the step in the x-strains is applied is 0.05 \textit{mm/s}.

Figure 5

Recording of x-force (upper tracing) and y-force (lower tracing) as a function of time in experiment 3. At t=170 min a step in the x-strain of 3 % is imposed and at t= 350 min an additional step of 6 % is imposed. The y-strain is constant. The external salt concentration is physiological except in the periods between t=40 min and t=145 min, between t=210 min and t=320 min and in the period t=380 min and 410 min in which the external salt concentration is zero. The thickness of the sample is between 1 \textit{mm} and 1.1 \textit{mm}. The sample is taken from the outer annulus fibrosus of the same disc as the sample of experiment 2. The clamp speed at which the step in the x-strain is applied is 0.05 \textit{mm/s}.
<table>
<thead>
<tr>
<th>sample number</th>
<th>thickness (mm)</th>
<th>origin</th>
<th>force tracings</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>$1 \geq \Delta z$</td>
<td>center annulus</td>
<td>Figs. 2 and 3</td>
</tr>
<tr>
<td>2</td>
<td>$\Delta z \approx 1$</td>
<td>outer annulus</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>3</td>
<td>$1.1 \geq \Delta z \geq 1$</td>
<td>outer annulus</td>
<td>Fig. 5</td>
</tr>
</tbody>
</table>

Table 1: Samples tested in the biaxial testing device.
Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 5: