A needle micro-osmometer for determination of glycosaminoglycan concentration in excised nucleus pulposus tissue

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Abstract

Purpose Aggrecan is one of the major macromolecular components of the intervertebral disc (IVD) and its loss is an early sign of degeneration. Restoration of aggrecan, and hence of biomechanical properties, is a major objective of biological therapies. At present, assessment of aggrecan concentration via its glycosaminoglycan (GAG) content is accomplished using biochemical and histological methods which require sacrifice of tissue. A minimally invasive method for assessing GAG, and hence aggrecan, which can avoid destruction of tissue, would be of benefit.

Methods We have developed a needle micro-osmometer that is capable of measuring flux of saline into excised human nucleus pulposus (NP) tissue. Using the isotropic osmotic stress technique to assess the swelling pressure of the excised NP tissue and assuming negligible collagen tensile stress, we were able to relate the flux to the tissue fixed charge density (FCD). GAG concentration is evaluated from its FCD via the radioactive tracer technique.

Samples representing different ages (28–59 years) and degeneration grades (1–4) were analyzed.

Results The flux is controlled by both the osmotic pressure difference across the probe's semi-permeable membrane and by the tissue permeability. A linear correlation was found between flux and the tissue FCD. The equation describing the linear fit is $FCD/(\text{total tissue hydration}) = 1.97 \times 10^{-4} + 8283 \times \text{flux}$ ($R = 0.836$, $p < 10^{-4}$). Thus, by measuring saline flux, the concentration of GAG can be determined.

Conclusions Micro-osmometry provides a reliable and minimally invasive tool for assessing GAG content in excised NP tissue. This method may be usefully applied in tissue engineering applications. It may also be useful for in vivo measurements if the question of the degenerative effect of needle puncture can be overcome.

Keywords Swelling pressure · Microdialysis · Intervertebral disc (IVD) · Fixed charge density (FCD) · Micro-osmometry

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Introduction

Physicochemical characterization of the intervertebral disc (IVD) continues to present a challenge because of its complex, multi-component structure. The central region of the IVD, the nucleus pulposus (NP), comprises a hydrated cartilaginous tissue composed mainly of negatively charged glycosaminoglycan (GAG)-substituted proteoglycan (PG) molecules, embedded within a collagen matrix. Aggrecan is the major PG in IVD and is responsible for much of the water-retaining properties of the disc, conferred through its attached sulphated GAG chains [1]. The high fixed charge density (FCD) of the GAG results in

osmotic imbibition of water [2, 3]; equilibrium water content is governed by the balance between PG osmotic pressure and resisting physiological loads [4, 5]. Hence, the osmotic pressure in the disc has to be sufficient to balance pressures which occurs in vivo [6–8]. In normal discs, PG comprises 5–15 % of the tissue by weight; at these concentrations, the equivalent osmotic pressures are ~ 0.05 – 0.3 MPa [9]. In ageing and degeneration, GAG concentration falls [10]; the resulting decrease in osmotic pressure leads to reduced disc water content, loss of height and possible pathological symptoms. Hence, detection of these changes is of significant diagnostic benefit both in vivo and in vitro. Previous in vitro studies of the osmotic pressures of tissue or of PG or GAG solutions used equilibrium dialysis [9, 11, 12] or sedimentation equilibrium methods [13]. These results were generally obtained via lengthy equilibration between the GAG molecules and well-calibrated macromolecular osmolytes such as polyethylene glycol (PEG) [9, 11].

Techniques which have been used to determine GAG concentration include both in vivo and in vitro methods. Conventional magnetic resonance imaging (MRI) techniques provide excellent detection of late-stage degenerative changes [14], but not of early degenerative changes in the matrix of IVD [15]. Delayed gadolinium enhanced MRI (dGemric) and Na-MRI have been used to quantify PG in articular cartilage [16–18]. However, deGemric is of limited usefulness in disc due to low diffusivity [19], whereas Na-MRI is limited by low spatial resolution. Johannessen et al. [20] demonstrated that T1-rho is linearly related to sulfated-GAG content ($r = 0.7$) in cadaveric human spine; however, the sensitivity was fairly low. In addition, MRI equipment is costly and not generally available. In vitro methods to determine GAG concentration include biochemical analyses of GAG, using, e.g. dimethyl methylene blue (DMMB) [21] or the radioactive tracer technique, employing radioisotopes [2]. Although accurate, these techniques require that the tissue be sacrificed.

The objective of the present study was to develop a minimally invasive and readily accessible method to determine GAG concentration in excised NP tissue without sacrificing the tissue. We investigated the use of a one-way microdialysis probe equipped with a sub-mm-sized semi-permeable membrane which functioned as a micro-osmometer. We determined the rate of fluid flow (flux) into or out of the probe upon insertion into the tissue as a measure of tissue osmolarity, arguing that the rate of flow depended on the difference in osmotic pressure between the tissue and the probe fluid as well as on the tissue permeability. The probe was initially calibrated against PEG solutions of known osmolarities. Flux was then measured for excised human NP tissue obtained from different ages (28–59 years), spinal levels and degeneration degrees

(1–4). We show that by measuring the flux into the tissue via the probe, FCD can be estimated, suggesting that—within the physiological range—this parameter is rapidly accessible.

Materials and methods

Tissue sampling

Tissue from lumbar discs was obtained post mortem or during routine anterior fusion surgery and stored frozen at -20 °C. Details of discs obtained (age, sex, spine level and grade) are given in Table 1. Tissue was graded by MRI according to the Pfirrmann scale [22]. Discs were first thawed, and the NP was separated from the annulus fibrosus (AF). Each NP slice was divided into two: one half was used for swelling and flux measurements using the micro-osmometer and the other half was further divided and used for measurements of FCD.

The study was approved by the Technion Research Ethics Committee, and was conducted according to the Ethical Principles stated in the Helsinki Declaration.

Force balance

With increase in applied pressure (mechanical or osmotic), the FCD, and hence the swelling pressure, increases as a result of water loss. This is true for both intact cartilaginous tissue and extracted PG [5, 23, 24]. In articular cartilage at equilibrium, the swelling pressure of PG is balanced both by the applied pressure, represented by the osmotic pressure of PEG, and by the collagen tension [5, 25]. For IVD, the stress exerted by collagen is insignificant throughout the broad spectrum of osmotic pressures [23, 24]. This implies that at equilibrium, the swelling pressure of PG in IVD equals that of the externally applied osmotic pressure.

Table 1 Profiles (age, sex, spinal level and degeneration grade) and fixed charge density (FCD) of excised disc tissue (NP) used in this study

Age (years)	Sex	Spinal level	Degeneration grade	FCD (mEq/g dry tissue)
28 ^a	F	L2–L3	1–2	0.711 ± 0.1
50 ^a	M	L2–L3	2–3	0.60 ± 0.1
59 ^a	F	L2–L3	2–3	0.49 ± 0.05
41 ^b	F	L4–L5	4	0.515 ± 0.1
34 ^b	M	L5–S1	2–3	0.403 ± 0.06
40 ^b	M	L5–S1	3–4	0.48 ± 0.05

^a Post mortem specimens

^b Surgical specimens

Fixed charge density (FCD)

FCD of excised NP tissue was measured by a radioactive tracer technique [2], using Na^{22} and Cl^{36} (Amersham, USA), with equilibration against 20 g PEG per 100 ml saline. Values of FCD per dry tissue weight are given in Table 1.

Swelling pressure

Variation of tissue hydration as a function of applied osmotic pressure was measured by equilibrium dialysis at 4 °C. Disc specimens were placed in dialysis tubing (MW cut-off 1000, Spectra/Por, USA) and immersed in calibrated PEG 20,000 (Merck, Germany) solutions of 5–25 g PEG per 100 ml saline, with equivalent osmotic pressures between 0.03 and 0.69 MPa [11, 26, 27]; after 48 h dialysis, equilibrium hydration was achieved. The samples were then removed from the dialysis tubing and were immediately placed in pre-weighed vials. These were weighed, the tissue samples were dried and the overall hydration was reported as: $W = (\text{wet weight} - \text{dry weight})/\text{dry weight}$ [28]. FCD on the basis of total tissue water (FCD/TTW) was obtained by dividing FCD (meq/dry weight) by W .

Probe setup and calibration

The needle micro-osmometer setup (Fig. 1) includes two semi-permeable compartments: (1) an external compartment in which the disc tissue is in equilibrium with the load-inducing PEG solutions via a semi-permeable membrane and (2) the probe, which consists of a small inner semi-permeable compartment (MAB-4, Microbiotech/se, Sweden). The outer diameter of the probe is 240 μm and it can be guided into the tissue using needles as small as 26 gauge (outer diameter of $\sim 450 \mu\text{m}$). Needles are used only for guiding and positioning, leaving the probe in direct contact with the tissue. The inner 6 kDa cut-off membrane, which is also sterilizable, was chosen to prevent diffusion of PEG molecules, aggrecan or collagen fragments into the probe. The probe was first soaked for 30 min in doubly deionized water (DDW), washed with saline via a PVC tube (ID 380 μm) (Technico, USA) connected to the inlet, and filled to the marked sign (fluid front) while the outlet was kept closed. The probe was calibrated at room temperature against a broad range of PEG solutions (5–25 % w/v in 0.5 mL saline), exerting osmotic pressures between 0.0255 and 0.608 MPa [27, 29]. The distance moved by the fluid front with time was measured with a caliper and the flux was defined as distance per unit time. The distance was converted to volume (1 mm $\rightarrow$ 0.1134 μl) using the cross-sectional dimensions of the PVC tube connected to the probe's inlet (Fig. 1). A linear flow with time was recorded for over 30 min, the slope being proportional to the

concentration of the PEG solution. Prior to tissue measurement, all probes were first calibrated against PEG solutions; only probes which deviated $<5\%$ from the calibration curves were used. Following calibration, the probe was washed (with open outlet) with saline and immersed in DDW until used.

Measurement of flux into excised NP tissue under externally applied osmotic pressure

Excised, lyophilized NP specimens ($\sim 5 \times 5 \times 3 \text{ mm}^3$) were thawed, weighed, sealed in dialysis tubing and immersed in PEG solutions at ambient temperature until equilibrium hydration was reached. The probe, filled with saline to a constant volume (Fig. 1), was guided into the tissue (4 mm depth) using a 22 gauge needle. For each tissue sample, the distance moved by the fluid front was measured manually as a function of time, and the flux during 5 min, calculated. Measurements were carried out in triplicate. Because GAG concentration and hydration are uniform throughout the nucleus [9, 30], flux measurements were carried out in three randomly chosen directions and sites. Flux was determined by linear regression from the slopes of the fitted lines of distance versus time. Only regressions with $r^2 > 0.98$ were included in the analyses. Results for each NP sample are presented as mean $\pm$ SD.

Statistical analysis

Curve fitting and statistical analyses were performed with Origin (Microcal, Northampton, MA, USA). Results were considered statistically significant when $p < 0.05$.

Results

Effect of applied osmotic pressure on equilibrium water content

As the applied osmotic pressure increased, the total tissue water decreased (Fig. 2), thereby increasing the concentration of the macromolecules, FCD and tissue osmotic pressure until the latter balanced the external applied osmotic pressure. The relationship between pressure and hydration is not linear: rather, at high hydration, a small decrease in applied pressure leads to large increase in hydration, while at low hydration, the opposite is observed.

Calibration of the needle micro-osmometer

The saline-filled probe was calibrated against a broad range of PEG solutions. The volume of fluid lost from the probe

Fig. 1 Overview of the needle micro-osmometer. **a** A MAB-4 (Microbiotech/se) microdialysis probe, equipped with a 6 kDa cut-off polyethersulfone semi-permeable membrane (4 mm in length and 240 µm outer diameter), was used as a one-way needle micro-osmometer (outlet was closed); **b** the probe was first filled with saline to a constant volume, guided into the excised NP tissue via a 22 gauge

needle and measurement of fluid movement with time was carried out; **c** prior to measurement, the tissue had been equilibrated via dialysis against well-calibrated PEG solutions and it remained in the dialysis tubing during the measurement to maintain equilibrium and prevent dehydration

increased linearly with time; data are shown for the first 5 min of measurement (Fig. 3). The flux increased with increase in PEG concentration (i.e. as osmotic pressure increased).

Measurement of flux under externally applied osmotic pressures

When a saline-filled probe is inserted into the excised NP tissue, saline begins to flow into the tissue across the microdialysis membrane. Samples of excised NP were equilibrated against different PEG solutions as described above. Microdialysis probes were then guided into each tissue slice using a 22 gauge needle and the flux measured.

Measurements of fluid loss from the probe (i.e. distance moved by the fluid front) as a function of time for the tissue specimens under different applied loads are presented in Fig. 4. Flux was determined by linear regression ($r^2 > 0.98$). Limiting the time of measurement to 5 min resulted in imbibition of less than 3.8 % of the total tissue water, thus minimizing perturbation of the local tissue hydration.

The values of flux measured during the first 5 min for the excised NP samples as a function of applied osmotic pressure are shown in Fig. 5a. Higher values were obtained for PEG solutions as compared to the excised NP samples. By averaging the flow rates for the different samples, a curve of mean flux versus applied pressure for the

Fig. 2 Pressure dependence of the hydration of excised NP tissue. The applied pressure is determined by the concentration of the PEG solutions with which the samples had been equilibrated. Each symbol represents a different donor

Fig. 3 Calibration curves of the micro-osmometer probe. The probe, filled with saline, was inserted into PEG solutions at room temperature and movement of the fluid front tracked. For each PEG concentration, the position of the moving fluid front was recorded for 5 min and data were fitted by linear regression from which slopes were calculated. Each point represents an average of three measurements. All correlation coefficients were >0.98 . The distance was converted to volume based on the cross-sectional dimensions of the PVC tube connected to the probe's inlet, as follows: 1 mm distance moved is equivalent to $0.1134 \mu\text{l}$

excised NP tissue can be obtained (Fig. 5a). The mean flux can be related to pressure by fitting a second-order polynomial as:

$$\text{Flux} = 1.33 \times 10^{-5} + 4.63 \times 10^{-6} \times \text{pressure} - 2.61 \times 10^{-7} \times \text{pressure}^2 \quad (R^2 = 0.997, p = 0.002) \quad (1)$$

Fig. 4 Movement of the fluid front with time following insertion of the needle micro-osmometer probe into the excised NP tissue. For each excised NP sample, the position of the moving fluid front was recorded for 5 min and data were fitted by linear regression from which slopes were calculated. The slopes (for all, $r^2 > 0.98$) represent the flux (distance per unit time) due to differences in osmotic pressure across the probe membrane. Prior to the flux measurements, tissue specimens had been equilibrated at 4°C using well-calibrated PEG solutions and then allowed to reach room temperature. Pressures corresponding to the PEG solution used (5–25 %) were interpolated to their values at room temperature [27, 29]. At each time point, three measurements were performed. In some cases, the standard deviation was so small that the error bars cannot be observed. In this figure, representative data obtained for excised NP from two different donors (28 and 59 years) are presented

or

$$\text{Pressure} = -0.157 - 102527.06 \times \text{Flux} + 9.30 \times 10^9 \times \text{Flux}^2 \quad (R^2 = 0.998, p = 0.012). \quad (2)$$

Equations 1 and 2 provide relationships between the measured flux and the swelling pressure of excised NP samples as determined by the proposed micro-osmometry method. The results of Fig. 5a show that the swelling pressure of PG in unloaded tissue is higher than that of saline.

Using flux to determine FCD

In Fig. 5b, we show the values of FCD/TTW (meq/g water) for the excised NP samples as a function of applied pressure. The values of FCD/TTW were obtained as described above. GAG concentration (expressed as FCD/TTW)

Fig. 5 **a** Relationship between applied pressure and flux in excised NP tissue. The applied pressure is due to the osmotic activity of the PEG solutions with which the excised NP samples had been equilibrated. The fluid flux into PEG solutions is shown for comparison. Measurements were carried out for 5 min at room temperature. Each point is an average of three measurements. **b** The relationship between FCD/TTW and applied osmotic pressure. Variation of FCD/TTW (meq/g water) with applied pressure for the excised NP samples was measured at 4 °C as described in the “Materials and methods”. Each point represents an average of three measurements. **c** Relationship between saline flux into excised NP and FCD calculated on the basis of total tissue water content (TTW) as derived from **a** and **b**. Measurements were carried out for 5 min at room temperature. The equation describing the linear fit is $\text{FCD}/\text{TTW} = 1.97 \times 10^{-4} + 8283 \times \text{flux}$, $r = 0.836$, $p < 10^{-4}$

increased with increasing applied pressure (Fig. 5b) because of the expression of water (Fig. 2); the relationship between applied pressure and GAG concentration is approximately linear. Combining the data presented in Fig. 5a, b, the relationship of FCD/TTW and fluid flux into the excised NP samples can be constructed (Fig. 5c). The equation describing the linear fit is

$$\text{FCD}/\text{TTW} = 1.97 \times 10^{-4} + 8283 \times \text{flux} \quad (R = 0.836, p < 10^{-4}). \quad (3)$$

Equations (1–3) may be applied to unknown excised NP samples on condition that the apparatus used is the same as we describe here.

Discussion

Flux as an indicator of FCD in excised NP tissue

We have used a needle micro-osmometer probe to assess the concentration of GAG in excised NP tissue as monitored by the FCD. This assessment is based on the measurement of saline flux across the probe’s semi-permeable membrane, in conjunction with the isotropic osmotic stress technique and equilibrium dialysis with respect to PEG solutions. Swelling pressure-hydration curves of excised NP samples were determined (Fig. 2) and since the collagen tension in NP is negligible [23], the osmotic pressure of the PEG solutions gives a direct measure of the swelling pressure of GAG at equilibrium. We found that tissue hydration decreased in a polynomial fashion as pressure was raised, leading to an increase in the concentration of FCD/TTW with pressure as previously reported [23].

The saline-filled probe is at lower osmotic pressure (P_{saline}) compared to the tissue matrix (π_{PG}) into which it is introduced. Hence, due to the negative driving force ($P_{\text{saline}} - \pi_{\text{PG}} < 0$), fluid was imbibed by the tissue. As saline flowed in, the tissue began to move slowly towards a

new local equilibrium. However, due to the negligible amounts of fluid flowing into the tissue (less than 3.8 % of the total tissue water in 5 min), we can assume that the measurements were carried out under equilibrium conditions and that the overall hydration and FCD/TTW remained nearly unchanged. For all excised NP samples processed, a linear relationship of distance moved by the fluid front with time was recorded, from which the flux was derived. A polynomial relationship between flux and swelling pressure was determined (Fig. 5a).

The flux into the excised NP samples is determined not only by the pressure difference across a membrane, as described by Darcy's law and by the hydraulic permeability of the probe membrane, but also by the permeability of the tissue which acts to restrain the flow of saline into the tissue [31]. The permeability is sensitive to the porosity of the tissue [32]. The tissue porosity is not constant but depends on the water content, which is itself a function of the applied pressure, here exerted osmotically by the PEG solutions but physiologically exerted by body weight and muscle activity [6, 33]. Therefore, osmotic or mechanical pressure has two opposite effects: it promotes more rapid flow across the micro-dialysis membrane as the pressure difference increases, but at the same time it reduces the tissue permeability by reducing its hydration. This is clearly shown by noting the marked reduction in the flow rate into the excised NP samples as compared with the PEG solutions (Fig. 5a). The small differences among the NP samples could thus be due to differences in overall porosity. By combining the measurements of (1) NP hydration and the resulting FCD/TTW as a function of applied osmotic pressure (Figs. 2, 5b) and (2) saline flux as a function of applied pressure (Figs. 4, 5a), we obtained a linear correlation between measured flux and FCD/TTW (Fig. 5c). Thus, the flow rate of saline into the excised NP samples, as measured by the micro-osmometer, provides a practical, minimally invasive means for assessing tissue FCD.

Possible application of the micro-osmometer technique in articular cartilage

Articular cartilage and IVD have very similar compositions [34]; however, the role of collagen is different in these tissues. Whereas in disc nucleus, collagen tensile pre-stress is negligible [23], for unloaded articular cartilage in contact with aqueous solution, the collagen network exerts significant tensile force to balance the osmotic pressure of the PG [11]. Therefore, extending the *in vitro* use of the proposed micro-osmometer to cartilage is possible only if the contribution of collagen tension to the force balance is included over a wide range of tissue hydrations.

Further development of the micro-osmometer technique

The principles of the proposed micro-osmometer have been demonstrated on a relatively small number of NP specimens. Obviously, further validation is required for a larger set of samples as well as for specimens from other cartilaginous tissues such as meniscus. For measurements in tissue from the AF, the direction and location of measurement must be taken into consideration due to the directionally dependent gradients of FCD, hydration [28] and external loading patterns. Even though the *in vivo* forces on the nucleus are directional, pressures in the nucleus are hydrostatic and the composition appears uniform, so similar considerations would not apply. Nevertheless, the use of the probe *in situ* in intact discs has not been tested and such measurements would require additional testing and validation. Another consideration, before the probe may be used *in vivo*, is possible damage arising through insertion of the micro-osmometer. The dimensions of the probe and guiding needles (as small as 26 gauge) were chosen to minimize injury to tissue.

Previous study using porcine or human discs showed that the degree of disc injury increased with the diameter of the needle and that a spinal needle smaller than 22 gauge is recommended to prevent post-surgery leakage [35].

In a rat IVD caudal model, injury due to needle puncture using 21, 25 and 30 gauge needles was shown to affect tissue biomechanics [36]. However, in another study using the same animal model, only 18 gauge needle punctures led to significant changes in disc biomechanics; these changes were not seen in 22- and 26-gauge punctured discs [37].

In fact, *in vivo* studies have now found that annular puncture during discography appears to induce disc degeneration in the long-term [38, 39]. The method presented here thus seems more appropriate for *in vitro* testing until complete safety is assured or until the annular injury is proved repairable [39]. Repair and regenerative therapies for the AF are being developed [40]; this will provide strong support for cell-based NP therapies currently under study [41, 42].

Measurement of osmotic pressure in tissue-engineered constructs is another possible application of the proposed needle micro-osmometer. It permits monitoring of GAG content during the deposition of engineered tissue during culture, without sacrificing the growing tissue and thus eliminating the need for lengthy, costly and destructive biochemical and histological analyses. Flux magnitudes measured in culture would presumably lie between the values found for PEG solutions and those of the excised NP specimens. As the tissue grows and more matrix is deposited, FCD, and hence the osmotic pressure, will increase towards the values found for disc tissue. For tissue

engineering applications, calibration solutions other than saline should also be considered (e.g. medium, PBS).

Conclusions

The needle micro-osmometer described above appears to be a convenient tool for in vitro determination of GAG concentration, as measurements using this device can provide rapid information on factors influencing turgor in pathological tissue. This device may also prove useful for tissue engineering applications by rapidly providing measurement of GAG content and thereby avoiding lengthy, costly and destructive biochemical or histological analyses.

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Conflict of interest None.

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