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Osmosis and viscoelasticity both contribute to time-dependent behaviour of the intervertebral disc under compressive load: A caprine in vitro study

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ABSTRACT

The mechanical behaviour of the intervertebral disc highly depends on the content and transport of interstitial fluid. It is unknown, however, to what extent the time-dependent behaviour can be attributed to osmosis. Here we investigate the effect of both mechanical and osmotic loading on water content, nucleus pressure and disc height. Eight goat intervertebral discs, immersed in physiological saline, were subjected to a compressive force with a pressure needle inserted in the nucleus. The loading protocol was: 10 N (6 h); 150 N (42 h); 10 N (24 h). Half-way the 150 N-phase (24 h), we eliminated the osmotic gradient by adding 26% poly-ethylene glycol to the surrounding fluid. For 62 additional discs, we determined the water content of both nucleus and annulus after 6, 24, 48, or 72 h. The compressive load was initially counterbalanced by the hydrostatic pressure in the nucleus. The load forced 4.3% of the water out of the nucleus, which reduced nucleus pressure by 44(±6)%. Reduction of the osmotic gradient disturbed the equilibrium disc height, and a significant loss of annulus water content was found. Remarkably, pressure and water content of the nucleus pulposus remained unchanged. This shows that annulus water content is important in the response to axial loading. After unloading, in the absence of an osmotic gradient, there was substantial viscoelastic recovery of 53(±11)% of the disc height, without a change in water content. However, for restoration of the nucleus pressure and for full restoration of disc height, restoration of the osmotic gradient was needed.

1. Introduction

The intervertebral disc connects adjacent vertebrae, transfers loads and provides flexibility to the spine. The main loading direction is axial (Smit, 2002), and the daily loading of the spine reduces water content and disc height, which is restored at night (Botsford et al., 1994; Emanuel et al., 2015).Osmotic attraction of water is considered to play an important role in both withstanding daily loads and the disc height recovery at night (Schroeder et al., 2006). With aging, however, the delicate homeostasis in the disc gets disturbed, leading to degeneration. This is a process in which cell biology, matrix physiology and biomechanics influence each other in a vicious circle (Vergroesen et al., 2015). This has been shown to be the major contributor to low-back pain (Cheung et al., 2009; Livshits et al., 2011), the most disabling disease worldwide (Vos et al., 2013). As the loss of charged proteoglycans in the nucleus pulposus that drive the osmotic gradient is considered the first sign of degeneration (Buckwalter, 1995), it is important to understand the role of osmosis in deformation and recovery. Furthermore, investigation of the interaction between osmotic, mechanical and hydrostatic pressure can provide valuable input for spine models, which are used for numerous tasks (Schmidt et al., 2013).

The current view on the role of fluid flow in the mechanical properties of intervertebral disc has been grounded in the late 70's by pioneering work by Urban and Maroudas (Urban and Maroudas, 1979, 1981; Urban and McMullin, 1984). They hypothesized that the negative charges in the proteoglycan chains stimulate an influx of positive ions. The increase in ion concentration results in a higher osmotic potential in the disc. This attracts water

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into the disc, thereby building up pressure. This special form of osmosis is called the Gibbs-Donnan effect (Elkin et al., 2010). When an external force is exerted on the disc, water is slowly pushed out of the disc. The velocity of the loss of water depends on the pore size of the matrix. The loss of water increases the osmotic pressure, as the ions are electrostatically bound to the proteoglycans and therefore remain in the disc. This continues until the osmotic pressure in the disc is in balance with the external pressure. Although the model is appealing, it is well-known that multiple time-dependent processes are occurring during loading and unloading of the intervertebral disc. Viscoelastic properties of the collagen fibres are likely contributing to deformation of the disc as well (Shen et al., 2011; van der Veen et al., 2013). However, it is difficult to separate the processes experimentally. The most straightforward way to do this would be to selectively eliminate one of these processes. Some studies tried to reduce the osmotic gradient by injecting substances that break down the proteoglycans (Detiger et al., 2013), but this also affects the hydraulic permeability of the tissue (Johannessen and Elliott, 2005). Another way to eliminate osmosis is to increase the osmolality of the fluid surrounding the disc. The aim of this study is to assess the role of osmosis in the biomechanical behaviour of the intervertebral disc. Therefore, we apply a simple loading protocol of static compression and subsequent unloading. When in equilibrium with the load, we will largely eliminate the osmotic gradient to visualize the effect of this gradient on the intradiscal hydrostatic pressure, disc height and water content of the nucleus pulposus and annulus fibrosis. Subsequently, the load is released without restoring the osmotic gradient. We hypothesize that the pressure, disc height and water content will decrease when the osmotic gradient is reduced, and that limited recovery is seen after unloading.

2. Methods and materials

Eighty-four lumbar intervertebral discs (13 T13-L1; 16 L1-L2; 13 L2-L3; 14 L3-L4; 13 L4-L5; 13 L5-L6, average transversal disc area $4.35 \pm 0.46\ cm^2$) were dissected from fresh-frozen mature caprine spines (age: 3–5 years) by parallel cuts through the vertebral bodies. Approximately 3.5 mm bone remained on each side of the discs, making sure that the entire disc is intact. The bone was rinsed and cleaned and inserted in a chamber filled with standard physiological saline (0.9% or 0.15 mol/l NaCl; Osmotic pressure: 731 kPa calculated using Eq. (1). Titanium porous platen on top of the endplates ensured that the fluid was completely surrounding the disc.

$$\Pi = c * R * T \tag{1}$$

With Π is osmotic pressure in kPa, c is the molar concentration in osm/l, R is the gas constant and T is the temperature in K.

2.1. Intradiscal pressure measurements

For eight intervertebral discs, the mechanical load was applied using a biomechanical testing device (model 8872; Instron and IST, Norwood, Canada) which allows precise application of the compressive load, as well as measurement of the changes in disc height by monitoring the displacement of the testing device. Furthermore, the hydrostatic pressure in the nucleus was measured using a 1.33 mm pressure needle (CTN-4F; Gaeltec devices Ltd, Dunvegan, Isle of Skye, Scotland), inserted through the lateral annulus until the tip of the needle was in the middle of the disc (see Fig. 1). All experiments were conducted in a temperature-controlled room at 20 deg Celsius. At t = 0, the discs were submersed in physiological saline, and an axial preload of 10 N was applied (see Fig. 2). At t = 6 h, the load was increased to 150 N,

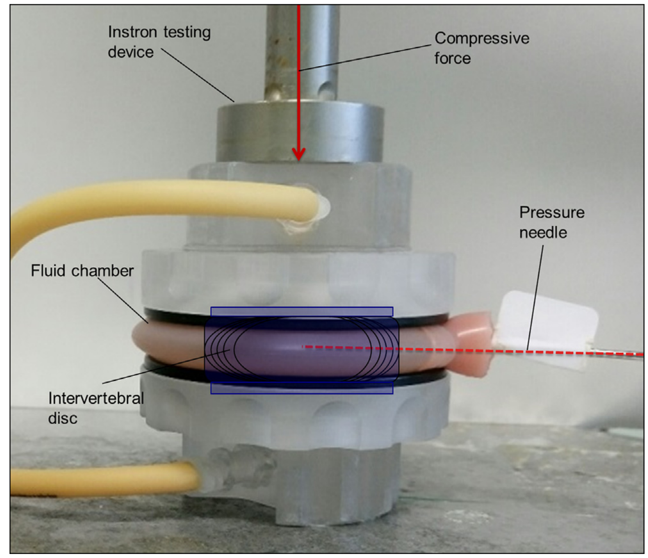

Fig. 1. Experimental setup.

which is estimated to be within a typical range for a goat during daily activities. 150 N is approximately 0.34 MPa, if the whole disc area is considered to carry the load. This static loading was held for 42 h. At t = 24 h, the osmotic gradient across the annulus fibrosus was largely eliminated by dissolving 26.6% Polyethylene glycol (PEG) in the surrounding medium, thus increasing the osmotic pressure by approximately 0.7 MPa (Michel, 1983; Money, 1989). At t = 48 h, the compressive pressure was released to 10 N again for an additional 24 h, while the osmotic pressure was kept high.

2.2. Water content measurements

Sixty-two additional discs were subjected to the same loading regime using a custom-build loaded disc culture system, which allows the change of culture fluid and axial compressive loading. Samples for water content measurements were taken at selected locations (Fig. 3) at t = 6 (n = 11), t = 24 (n = 17), t = 48 (n = 21) or t = 72 (n = 13) hours. After removal of the cranial end-plate with a surgical blade, a strip of approximately 5 mm wide was taken from side to side, and divided in 6 equal parts (see Fig. 3). The two outer sections (excluding the outermost lamella to avoid that wetness of the surface of the disc during dissection influenced results) will be referred to as annulus, the two inner sections as nucleus. The four tissue samples were removed with a blade and stored in a closed Eppendorfer tube with a pre-measured weight. Wet weight was measured with a Satorius scale (type R180D, Göttingen, Germany). After freeze-drying the samples with a speedvac, the dry weight was measured. Water content was calculated as shown in Eq. (2).

$$Water\ content = \frac{ww - dw}{ww} * 100 \tag{2}$$

With ww = wet weight (g) and dw = dry weight (g).

2.3. Extended protocol

To check whether the discs were still intact after the experiments and show complete recovery, in the final four discs the protocol was extended for an additional 24 h on norm-osmotic physiological saline. Also, for an additional 14 discs, water content was determined at t = 96 h after the extended protocol of 96 h.

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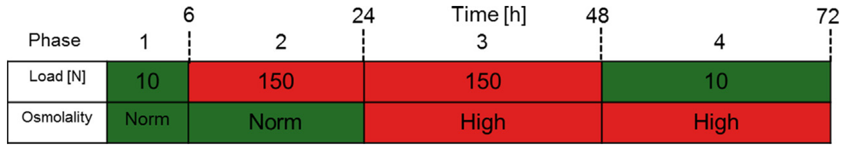

Fig. 2. Loading regime. Norm = physiological saline; High = 26.6% PEG added.

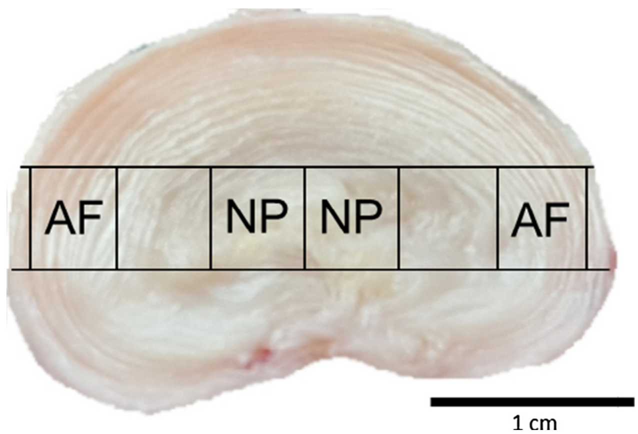

Fig. 3. Example of selection of samples. NP = Nucleus Pulposus, AF = Annulus Fibrosis.

2.4. Data analysis and statistics

Changes in nucleus pressure and disc height were analysed using custom-made MATLAB programs. Water content of the two samples of the structures of each disc were averaged. In case of very large difference between the two samples (more than 10% water content difference), both were discarded unless one was a clear outlier, defined as a deviation from the average of more than four standard deviations. Differences in water content between sequential time points were analysed, separately for the nucleus and annulus, using one-way ANOVA analysis in SPSS 22 (IBM, Armonk, NY). When found significant, post hoc testing for sequential differences in time was performed using a Bonferroni correction for multiple testing. Repeated measures ANOVA was performed on pressure and disc height at different time points.

3. Results

Over the time points, the ANOVAs showed significant overall differences in pressure, disc height and water content (all $p < .001$). Subsequent analyses were performed separately for phase 1–4 in the experiments (see Fig. 2).

Phase 1: preloading at 10N under normosmotic conditions ($t = 0–6$ h): Despite 10N compressive force, some discs initially showed an increase in pressure and disc height (Fig. 4). This part was used as preloading to avoid great differences in hydration due to handling. At t=6 h, the average nucleus water content was 75.3 ± 2.0%, and the average annulus water content was 63.2 (±3.0)%.

Phase 2: loading at 150N under normosmotic conditions ($t = 6 – 24$ h): It is interesting to notice that, although this is a creep experiment, the response of the nucleus pressure to the increased compressive force is one of a stress-relaxation type. After the load was increased, the hydrostatic pressure initially increases to an average peak pressure of 1308 ± 173 kPa. Thereafter, the pressure slowly reduces to an average of 56 ± 6% of the peak value. This process is faster than the loss in disc height, which still showed creep at t = 24 h (Fig. 4). The external load of 150 N forced 4.3% of the water out of the nucleus, but no loss of water content in the annulus was found.

Phase 3: loading at 150 N under high osmotic conditions ($t = 24 – 48$ h): Subsequently, the equilibrium disc height was disturbed by the elimination of the osmotic gradient. As a result, a significant ($p < .001$) loss of annulus water of 5.6 ± 0.9% was found, while nucleus pressure and water content remained unchanged. This is surprising, as the nucleus is regarded as a high-osmotic vessel, with the annulus and endplate as a confining membrane. In contrast to this idea, the osmolality of the surrounding fluid affected the annulus, rather than the nucleus. Thus, disc height was lost due to fluid loss in the annulus, without loss of nucleus water content or pressure.

Phase 4: unloading to 10N under high osmotic conditions ($t = 48 – 72$ h): After unloading, 53(±11)% of the disc height loss after t = 6 h was restored without a change in water content in either structure. Still, the nucleus pressure remained close to zero. This means that significant restoration of disc height can be achieved without water intake, and without nucleus pressure driving the restoration of disc height.

Phase 5 (extended protocol): loading at 10N under normosmotic conditions ($t = 72 – 96$ h): To check whether our protocol had damaged the intervertebral disc, in four discs the test was extended with another day of normosmotic loading at 10 N. An example can be seen in Fig. 5. Both hydrostatic pressure and the disc height restored towards t = 6 h values. In 14 discs, the water content was measured at 96 h as well, and showed restoration of water content to t = 6 h values (nucleus 76.9 ± 2.0%; annulus 65.2±2.0%, no significant difference to t=6 h (nucleus $p = 1.00$ & annulus $p = .56$)). This indicates that the intervertebral disc was not damaged.

4. Discussion

In this study we investigated the complex relation between external load, osmosis, hydrostatic pressure and water content in the intervertebral disc. The initial increase in hydrostatic pressure in the nucleus at 10 N loading, followed by a slow reduction (phase 2) at 150 N loading, is in line with previous findings (Galbusera et al., 2011; Vergroesen et al., 2014). This indicates that, after increase in load, the pressure is initially counterbalanced by the hydrostatic pressure in the nucleus. However, as the disc is in a quasi-static state, the loss of pressure over time means that the load is either distributed over a larger surface, or that the extracellular matrix is carrying more load by stiffening. The latter is supported by the increasing stiffness found with dehydration (Bezci et al., 2015).

Interestingly, under 150 N load in normosmotic medium (phase 2), the nucleus lost water, but the annulus did not. Remarkably, after the elimination of osmotic gradient at t = 24 h, annulus water content decreased, but no changes in nucleus water content or pressure were found. Based on the model of Urban and Maroudas (1979; 1981), we had expected a change in nucleus pressure and water content after eliminating this gradient, but this was not the case. This indicates that the equilibrium in the disc is not only determined by the osmotic gradient between the nucleus and surrounding fluid, but that the annulus also has major role in

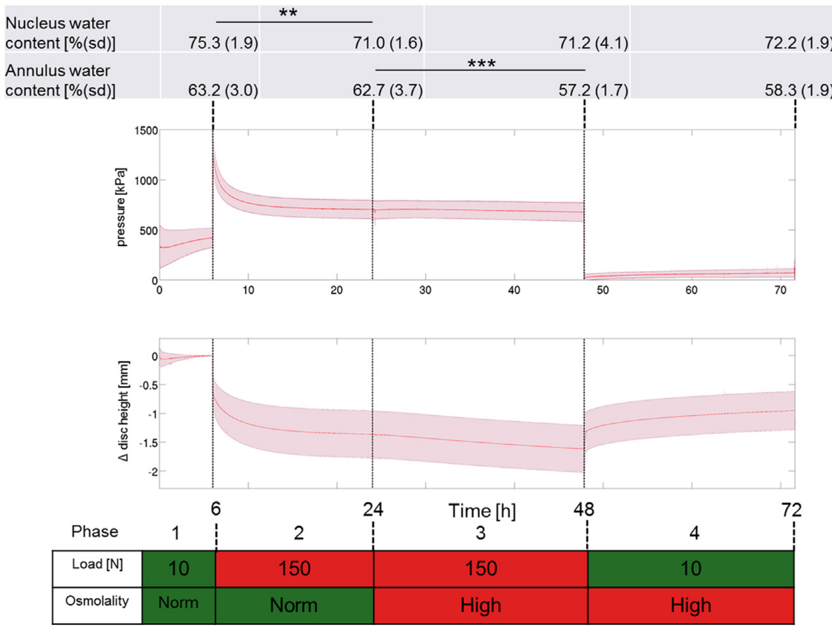

Fig. 4. Bottom: Average (±1 SD) of disc height relative to t = 6 h. Middle: Average (±1 SD) hydrostatic pressure, as measured with the pressure needle in the nucleus pulposus. Top: Water content for nucleus pulposus and annulus fibrosis at different time points. Asterisks indicate significant change between consecutive timepoints. $^{**}p < .01$, $^{***}p < .001$.

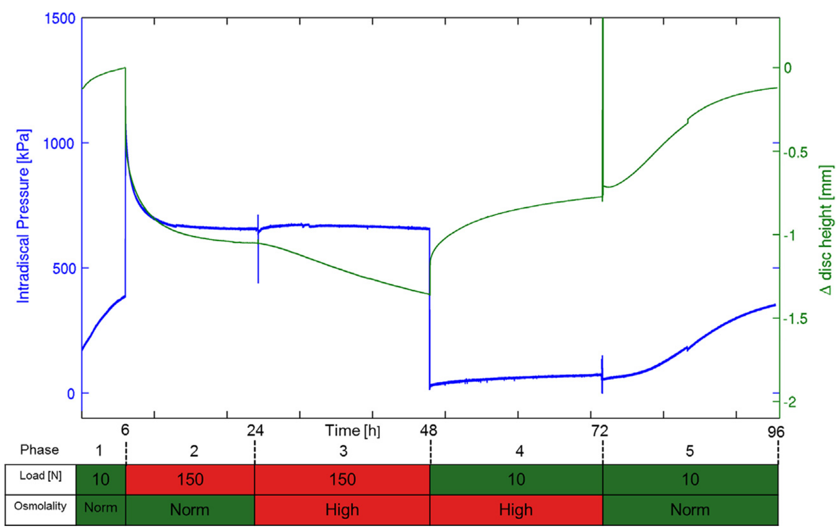

Fig. 5. Typical example of an extended protocol. Intradiscal pressure is shown in blue, changes in disc height compared to t = 6 h is shown in green. The extended protocol was implemented to check whether the intervertebral discs would return to initial state after unloading in an normosmotic environment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the osmotic balance. Furthermore, this indicates that the pressure in the nucleus pulposus is not directly affected by the osmotic gradient with the surrounding fluid when the nucleus is compressed, in contrast to the recovery phase. Possibly, a large fraction of the water molecules are bound to the proteoglycans, which has been suggested based on magnetic resonance imaging (Chiu et al., 2001). The compressive load may remove most free water from the nucleus pulposus, which may reduce osmotic effects. After unloading of the disc under high external osmolality, the disc height showed substantial viscoelastic recovery (phase 4). The water content did not change during this phase, neither in the annulus, nor in the nucleus; thus, as water flow was absent, we can attribute this recovery in this phase to viscoelastic behaviour. Moreover, nucleus pressure remained very small in this part of the experiment compared to the preload phase. This shows that osmotic water attraction is only in part responsible for disc height restoration during unloading and that viscoelastic behaviour of the annulus plays a prominent and possibly underestimated role. In the extended protocol after restoration of the osmotic gradient (phase 5), recovery of disc height, nucleus pressure and water content was observed. This visualizes that for full recovery of the disc height at night, the osmotic gradient is necessary. The restoration of the nucleus pressure was almost fully dependent on the osmotic gradient. This is important, as the cells in the nucleus are mechanosensitive and maximize their aggrecan production at ~0.3 MPa (Handa et al., 1997). Therefore, without the nucleus pressure, less aggrecan will be produced, which in turn leads to less osmotic gradient. This could lead to degeneration (Vergroesen et al., 2015).

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The reduction of the recovery due to increased medium osmolality (47%) was less than found in a dynamic loading protocol (57%, Vergroesen et al., 2017). This may be due to the shorter recovery period of 8 h, as the recovery was slower in a high-osmolarity group. Bezci and O'Connell (2017) also found reduced recovery when using bovine tail discs and 1.5M NaCl, indicating that increasing the salinity has the same effect on disc height. With static compression of rat tails, the nucleus pulposus lost most water, which agrees with our findings (Masuoka et al., 2007). However, in contrast to our findings, a significant loss of annulus water content was also found in that study, indicating a possible difference between lumbar and caudal discs. The annulus fibrosis GAG-content in the goat is approximately 4–6 times lower than in the nucleus pulposus (Vergroesen et al., 2017). Bezci et al., (2015) however reported that the swelling capacity of the annulus fibrosis is higher than expected based on GAG-concentration, and hypothesized that other extracellular matrix components, such as elastin, also contribute to the swelling capacity. The results from this study indicate that the annulus fibrosis swelling capacity is indeed of significant importance for maintaining disc height, despite the lower GAG-content. Future research on how different biochemical components contribute the swelling capacity of the annulus fibrosis could provide valuable information on the understanding of intervertebral disc mechanics.

The nucleus pulposus is regularly modelled as a biphasic structure containing a permeable solid and a fluid (e.g. Iatridis et al., 1998; Johannessen and Elliott, 2005). The osmotic and viscoelastic properties, however, should be considered to fully understand the biomechanical behaviour. Examples of models that consider osmotic properties are the osmoviscoelastic model (Schroeder et al., 2006) and a Fixed Osmotic Pressure model (Galbusera et al., 2011). The results of this study indicate that swelling of the annulus fibrosis should be incorporated as well, as it is a major attributor to the changes in disc height.

There is no consensus in the literature regarding the direction of fluid flow during loading and unloading. Ayotte et al. (2001) found a direction-dependent fluid flow through the endplate, and hypothesized that this is the main route for fluid transport. However, it has been shown that blockage of the endplate did not affect intervertebral disc creep (van der Veen et al., 2007), which suggests that fluid flow through the annulus is a major contributor. In that regards, it is interesting to observe that in the creep phase (phase 2) no loss of water in the annulus was seen. On the other hand, changing the osmotic gradient affected the water content in the annulus rather than the nucleus (phase 3). This suggests a role for annulus fluid flow in the osmotic balance.

One limitation of this study is that we considered only one load level. The forces used are based on *in vivo* pilot experiments with an instrumented cage in a goat (Dormans et al., 2004). This indicated 150 N to be in the range of daily activities. After application of 150N, the intradiscal pressure increases to 1.3 MPa, and then reduces in time to 0.7 MPa. When compared to the *in vivo* human data of Wilke et al. (1999), this range is higher than daily activities like walking (0.5–0.6 MPa) and sitting (0.46–0.56 MPa). Sato et al. (1999) found pressures between 215–904 kPa with upright standing and sitting. Furthermore, in mechanobiological studies by Paul et al. (2013, 2012), protocols with loading phases of 150N were found to be in the physiological range for goat lumbar intervertebral discs. Another limitation of this study is the use of goat spines. Both the initial water content and the amount of water expelled during the loading phase (4.3%) is lower than estimated in literature available for human intervertebral discs (Botsford et al., 1994). This could be due to interspecies difference, as previous measurements by our group showed a similar water content in goat spines (Paul et al., 2012). Furthermore, the creep data cannot be quantitatively compared to experiments that used human or bovine samples. A potential source of bias in water content measurement is rehydration or drying during dissection of the tissue. The procedure took approximately 3 min from removal of load until insertion in the Eppendorfer tubes. Two samples were taken for both the nucleus an annulus of each disc, and we found no order effect in the water content, i.e. the annulus or nucleus sample that was dissected first had no significantly higher or lower water content. This implies that no fast drying or swelling was occurring during dissection. The tests were conducted at 20 deg Celsius, which may slightly underestimate any osmotic effects, as osmotic pressure is linearly related to absolute temperature (see Eq. (1)). Another limitation is that the pressure needle used had a non-negligible diameter of 1.33 mm. The average lumbar disc height of a goat is approximately 5.3 mm (Krijnen et al., 2006). It is unlikely that the needle directly interfered with the disc height loss, as pressure needle contact with bone would be visible in the pressure data. However, the volume of the pressure needle will increase the pressure (Bashkuev et al., 2016) although in equilibrium this effect may have largely disappeared. Furthermore, the volume of the needle does not influence the measurement of deviations in the pressure (Bashkuev et al., 2016), and the conclusions of this paper depends upon changes after equilibrium rather than absolute pressures. An additional limitation was that the check for recovery to the initial state of the pressure and disc height in phase 5 was only conducted with four discs. This hampers statistical analysis for the hydrostatic pressure and disc height. However, water content at 96 h was measured in 14 discs, and confirmed full recovery. Furthermore, previous experiments by our group did not find changes in mechanical properties or GAG-content after 3 weeks of a loading pattern with similar forces (Paul et al., 2012). Supported by the full recovery of water content after unloading, we assume that no changes have occurred in GAG-content in this short protocol either. Another limitation was that a protocol of static compression was used to make the experiment as easy to interpret as possible. However, this did not allow the measurement of changes in stiffness throughout the experiment, as dynamic measurements are needed for that. Based on results of Bezci et al., (2015) increase in stiffness with dehydration could be expected, and recovery upon rehydration. However, Vergroesen et al., (2017) found no relevant changes in stiffness related to osmolality of the medium.

No control groups were used, as the effect of osmotic gradient was determined with a within-subject analysis. This avoids the problems due to between-subject variability. The extended protocol was implemented to check that reinstalment of the osmotic gradient did bring the intervertebral discs back to original state. This confirmed that the effect on recovery was indeed caused by the reduced osmotic gradient. PEG was used previously to avoid swelling of nucleus explants (van Dijk et al., 2011). The concentration of 26.6% was based pilot testing in previous research, where diurnal axial dynamics was found to change severely with this concentration (Vergroesen et al., 2017). The advantage of the large (8000 w) molecules is that penetration of the disc can be avoided, which would have occurred if e.g. NaCl had been used instead. However, it was not intended to mimic physiological circumstances; the high-osmotic environment was only implemented to study the contribution of the osmotic gradient to the mechanical balance in the disc under compression.

In conclusion, the water content and viscoelastic deformation of the annulus play an important role in the axial biomechanics of the intervertebral disc. The recovery of disc height after unloading is partly viscoelastic, as significant recovery is seen without water uptake. However, for restoration of the nucleus pressure, and for full restoration of disc height, an osmotic gradient is needed.

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