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Abstract

The intervertebral disc faces high compressive forces during daily activities. Axial compression induces creeping fluid loss and reduction in disc height. With degeneration, disc fluids and height are progressively lost, altering biomechanics. It is assumed that this loss of fluids is caused by a drop in osmolality in the disc due to proteoglycan depletion. Here we investigate the isolated effect of a reduction in osmosis on the biomechanical properties of the intervertebral disc. Continuous diurnal loading was applied to healthy caprine intervertebral discs in a loaded disc culture system for a total of 6 days. We increased testing bath osmolality with two doses of polyethylene-glycol (PEG), thereby reducing the osmotic gradient between the disc and the surrounding fluid. This way we could study the isolated effect of reduced osmosis on axial creep, without damaging the disc. We evaluated: daily creep and recovery, recovery time-constants and compressive stiffness. Additionally, we investigated water content. There was a strong dose-dependent effect of PEG concentration on water content and axial creep behaviour: disc height, amplitude and rate of creep and recovery were all significantly reduced. Axial compressive stiffness of the disc was not affected. Reduction of water content and amplitude of creep and recovery showed similarity to degenerative disc biomechanics. However, the time-constants increased, indicating that the hydraulic permeability was reduced, in contrast to what happens with degeneration. This suggests that besides the osmotic gradient, the permeability of the tissues determines healthy intervertebral disc biomechanics.

Keywords (5):

Intervertebral disc; creep; osmolality; proteoglycans; permeability

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Abstract

The intervertebral disc faces high compressive forces during daily activities. Axial compression induces creeping fluid loss and reduction in disc height. With degeneration, disc fluids and height are progressively lost, altering biomechanics. It is assumed that this loss of fluids is caused by a drop in osmolality in the disc due to proteoglycan depletion. Here we investigate the isolated effect of a reduction in osmosis on the biomechanical properties of the intervertebral disc. Continuous diurnal loading was applied to healthy caprine intervertebral discs in a loaded disc culture system for a total of 6 days. We increased testing bath osmolality with two doses of polyethylene-glycol (PEG), thereby reducing the osmotic gradient between the disc and the surrounding fluid. This way we could study the isolated effect of reduced osmosis on axial creep, without damaging the disc. We evaluated: daily creep and recovery, recovery time-constants and compressive stiffness. Additionally, we investigated water content. There was a strong dose-dependent effect of PEG concentration on water content and axial creep behaviour: disc height, amplitude and rate of creep and recovery were all significantly reduced. Axial compressive stiffness of the disc was not affected. Reduction of water content and amplitude of creep and recovery showed similarity to degenerative disc biomechanics. However, the time-constants increased, indicating that the hydraulic permeability was reduced, in contrast to what happens with degeneration. This suggests that besides the osmotic gradient, the permeability of the tissues determines healthy intervertebral disc biomechanics.

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Introduction

Low back pain is a major health problem in western societies (Murray et al., 2015). Up to 40% of adult persons in the United States report low back pain in the past three months, and 20-33% of low back pain patients is unable to work (Jacobs et al., 2011; Lambeek et al., 2011; Murray et al., 2012), which causes significant direct and indirect socio-economic costs (Lambeek et al., 2011). Prevention and therapeutic intervention is hampered because the veritable cause of low back pain remains unclear; however, a relationship is present with intervertebral disc degeneration (Cheung et al., 2009; Jacobs et al., 2011; Wang et al., 2012). Intervertebral disc degeneration comes with a loss of water from the nucleus pulposus (Antoniou et al., 1996). This results in a clear change in axial intervertebral disc biomechanics when subjected to diurnal axial loading (Emanuel et al., 2015), which could cause low back pain by increasing strain on surrounding vertebrae, muscles, ligaments and facet joints (Pollintine et al., 2004).

According to the popular theory developed in the eighties by Urban & Maroudas (Urban and Maroudas, 1981) axial disc biomechanics are predominantly driven by the osmotic gradient between the nucleus pulposus and the fluid surrounding the disc. This osmotic gradient is caused by the negative charges of the proteoglycans which attract positive ions into the nucleus pulposus, known as the Gibbs-Donnan effect (Urban et al., 1979). These ions cause an osmotic pressure, which pulls water into the disc when the disc is unloaded, such as during night time rest. When the disc is loaded, water is pushed out of the disc, which increases the concentration of ions in the disc, and thereby the osmotic pressure. Thus, osmotic pressure maintains tissue hydration and prevents the disc from complete collapse. Although the presented theory has a wide scientific basis, fundamental experimental data on the effect of a reduced osmotic gradient on diurnal intervertebral disc biomechanics is lacking. This is needed in order to develop therapies to restore disc biomechanics in degenerated discs.

The first sign of degeneration is a loss of proteoglycans, water and disc height (Adams and Roughley, 2006; Antoniou et al., 1996). The loss of proteoglycans disturbs the osmotic balance, which may be the cause of the water loss and biomechanical changes found with degeneration. In previous research, it was established

that the loss of proteoglycan content was indeed associated with changes in biomechanical properties *in vitro* (Emanuel et al., 2015). Concurrently, altered hydration of the intervertebral disc changes biomechanical properties *in vitro* (Costi et al., 2002). Apart from the loss of proteoglycans in the nucleus pulposus, the annulus fibrosus and cartilaginous endplates are also damaged during degeneration —and thus may be less confining or more permeable— (Brinckmann and Horst, 1985; Perie et al., 2006), this could also influence axial biomechanics of the intervertebral disc. Therefore, we aim to isolate the effect of reduction in osmotic gradient on diurnal disc height changes.

In previous research we found that reducing the salinity of the testing fluid increases the disc height under a range of static loads (Vergroesen et al., 2016), which implies a relation between osmolality, water content and axial biomechanics. Consequently, we hypothesized that daily fluid flow (*i.e.* disc height change) is dependent on the osmotic gradient (Vergroesen et al., 2016). However, when using a saline solution, the charged ions may enter the disc which may limit the effect on the osmotic gradient. In other research, the inert molecule poly-ethylene-glycol (PEG) has been successfully used to reduce swelling pressure of nucleus explants *in vitro* (Urban and Maroudas, 1981; van Dijk et al., 2011). Therefore, in this study, we increase the osmolality of phosphate buffered saline with PEG to attenuate the osmotic gradient, which presumably induces water and disc height loss from the intervertebral disc.

In this *in vitro* work we investigate the isolated effect of reducing the disc's osmotic potential on normal axial disc biomechanics under simulated physiological loading. In line with the theory of Urban and Maroudas (Urban and Maroudas, 1981) and our own hypothesis on diurnal fluid flow (Vergroesen et al., 2016), we hypothesize that the axial biomechanical behaviour will change with reduced osmotic gradient similar to degeneration. Thus, we expect to see a loss of disc height, reduced loading creep, decrease in time constants, and higher compressive stiffness (Emanuel et al., 2015).

Materials and methods

Specimens and specimen preparation

Twelve intervertebral discs of skeletally mature 3-to-5-year-old Dutch milk goats (2 spines, Th13-L1 to L5-L6) were obtained from a local abattoir, and stored at room temperature in PBS soaked gauzes (PBS, Gibco, ~285 mOsm) until experimentation. Within 12 hours of sacrifice, spines were rinsed with iodine solution, and soft tissue and posterior elements were removed under aseptic conditions. The discs were isolated by cranial and caudal parallel cuts, approximately 3.5 mm from the intervertebral disc, to include the endplates. Subsequently, endplates were rinsed using PBS until no haemoglobin was visible, to ensure that the endplates were open to allow fluid-flow.

We placed the intervertebral discs in our custom build loaded disc culture system at 37°C and 5% CO_2 (Paul et al., 2013, 2012). Discs were submerged in PBS supplemented with 10,000 u/ml penicillin, 250 mg/L streptomycin, and 1.5 mgr/mL amphoterizin B (PSF 1%, Sigma Aldrich, St. Louis, MO) to prevent infection. No culture medium was used, as the cell biology was not an outcome measure for this study. For certain groups, the osmotic value of PBS was increased using poly-ethylene-glycol (Sigma-Aldrich) with a molecular weight of 8,000 (van Dijk et al., 2011). Discs were stratified into three experimental groups (N=4 per group): testing on regular PBS (control, ~285 mOsm); testing on PBS supplemented with 13.3% w/v PEG (PEG 13.3, ~790 mOsm); or testing on PBS supplemented with 26.6% w/v PEG (PEG 26.6, ~1300 mOsm). Osmolality of the testing fluids was checked for all three conditions prior to testing (Osmomat 30, Gonotec, Berlin, Germany).

Mechanical loading

Continuous simulated physiological loading (1Hz sinusoidal, Figure 1) was applied for 6 days as previously described (Paul et al., 2012). In short, for 8h per day, low dynamic load of 40-60N was applied as night-time unloading, a daytime loading 16h load alternating in magnitude between 40-60N and 80-180N, for every 30 minutes. These loads generate mean intradiscal pressures of ~0.45MPa and ~1.05MPa, respectively (Vergroesen et al., 2014), reflecting pressures generated during normal diurnal activity in sheep (Reitmaier et al., 2013). For the evaluation of compressive stiffness, the final half hour of daytime loading and night time unloading consisted of 0.25Hz triangles at 80-180N and 40-60N, respectively. Due to

unloading between harvest and experimentation, we consider the first 24h of the experiment as day 0, and the subsequent five days are analysed for the experiment.

Biomechanical evaluation

All force and displacement data were recorded at 100Hz, and loaded into Matlab (version 2013a for Linux). After re-sampling to 20Hz, data was used to calculate characterising biomechanical parameters (Figure 2): (1) the overall disc height loss (mm) relative to the start of the experiment; (2) the creep during loading and (3) recovery (mm) during the unloading of the disc (note that the instant disc height change during the first minute of loading and unloading is not included because this is predominantly the result of elastic tissue deformation, rather than fluid-flow (O'Connell et al., 2011; van der Veen et al., 2008)); the rate of recovery during (4) the first half hour (start), (5) the last half hour of the unloading in the 'night' (end, all mm/h); and compressive stiffness during the end of (6) the recovery phase and (7) the creep phase (N/mm). Additionally, the difference between the creep and the recovery was used as a measure for the general disc height loss (if there is more creep than recovery, the disc loses height over the days). Finally, time constants (hours) were obtained using a double Voight model (equation 1) fitted using a least-squares method on the displacement curves for the unloading phases for each day, the long-term time constant τ2 was used (van der Veen et al., 2013).

1. $$x(t) = L\left[\frac{1}{S_1}\left(1-e^{-(t/\tau 1)}\right)+\frac{1}{S_2}\left(1-e^{-(t/\tau 2)}\right)+\frac{1}{S_E}\right]$$

Water content and quantitative biochemistry

After six days, directly following the experiments, tissue was processed for assessing water content and for quantitative biochemistry. Care was taken to prevent water uptake during processing, by using a specially designed double bladed hand saw. The intervertebral disc was divided into nucleus pulposus (NP), and outer annulus fibrosus (AF). Wet weight was measured, tissue was freeze dried (Speedvac, Thermofisher, Waltham, MA, USA), and dry weight was measured. Wet and dry weights (WW and DW) were used to determine water content using the formula: $(WW-DW)/WW$. Dry weight samples were digested in a papain-digestion buffer as pre-processing for DMMB assay for glycosaminoglycan content *in duplo*

(Biocolor Ltd., Carrickfergus, UK), and DMBA assay for hydroxyproline content *in triplo*, as previously described (Hoogendoorn et al., 2008; Paul et al., 2012).

Statistical Analysis

To analyse the effects of experimental group and day, SPSS (IBM Software, Armonk NY, USA, version 20 for windows) was used to perform Huynh–Feldt's repeated measures ANOVA, with experimental group as between subjects factor, and with day (five levels, or six levels for loading creep) as within-subject factors. Post-hoc Tukey HSD tests were used to test the differences between the experimental groups.

For biochemistry a repeated measures ANOVA was used with experimental group as between subjects factor, and with disc tissue (2 levels) as within-subject factor. All values are expressed in graphs as mean +/- SD. A p-value <0.05 was considered statistically significant.

Results

Water content and quantitative biochemistry

Testing in fluids with PEG reduced water content (Figure 3a, group effect, $p<0.0001$), in a dose-dependent manner (post-hoc p-values<0.017). Overall, water content was lower in the annulus than in the nucleus (tissue effect $p<0.0001$), with a relatively larger decrease of water content in the annulus with higher PEG concentrations (tissue*group-interaction: $p=0.028$). Glycosaminoglycan and hydroxyproline content per dry weight were not affected by testing in fluids with PEG (Figure 3b-c, group effects $p=0.785$, and $p=0.767$, respectively) which supports the notion that intervertebral discs are not damaged before or during the experiment.

Biomechanical evaluation

There was a visible dose-dependent effect of osmolality of the testing fluids on the diurnal changes in disc height (Figure 4). Unloading recovery was affected by group (Fig 5a, group effect $p<0.0001$, post-hoc $p<0.0001$), and remained unchanged over days. There was a strong interaction between group and day (Fig

5b, group effect $p<0.0001$): the loading creep was higher in de PEG-groups the first day and smaller in subsequent days (post-hoc $p<0.0001$). At day 5, loading and unloading creep were in equilibrium, and general disc height remained similar between days 4 and 5 (Figure 5c, post-hoc day 4 vs day 5 $p=0.464$), which indicates a dynamic equilibrium (Emanuel et al., 2015). There was a dose-dependent loss in dynamic behaviour of the intervertebral disc in both PEG groups: loading creep, and unloading recovery were both reduced with increasing testing fluid osmolality (Fig 5a-c, group effect, $p<0.0001$). Compressive stiffness increased over the days for all groups during unloading, but was not affected by experimental group, neither for the loading nor for the unloading phase (Figure 6, group effects: $p=0.575$ and $p=0.433$, respectively).

Biomechanical evaluation – recovery phase

A more detailed analysis of the recovery patterns revealed that the rate of recovery of disc height during the first half hour of the unloading phase was reduced for both PEG groups (Figure 7a, group effects $p<0.0001$). Conversely, during the last half hour of the unloading phase, the PEG 13.3 group had the highest rate of recovery (Figure 7b, group effect $p<0.033$), indicating recovery was not yet complete in this group. The time constant of the recovery phase increased over the days of the experiment (Figure 7c, effect of day, $p<0.003$). At day 5, there was a dose-dependent increase in time-constants with increasing PEG concentration (group effect, day 5 $p=0.048$).

Discussion

This study investigated the effect of reducing the osmotic gradient between the intervertebral disc and the surrounding fluid on the biomechanical behaviour of the disc. The water content was reduced with increasing concentration of PEG. This showed that the osmotic gradient was indeed reduced in discs with a similar biochemical composition. We found a strong dose-dependent effect of the osmolality of the testing fluid, resulting in more general disc height loss, slower recovery, longer time-constants and less daily creep. In contrast, compressive stiffness of the intervertebral disc was not affected.

The reduction of disc height, recovery and creep found in this study closely resembles the biomechanical changes found in human lumbar discs under simulated physiological axial loading (Emanuel et al., 2015).

Furthermore, a decrease in water content and general disc height loss are key features of degeneration (Adams and Roughley, 2006). Inversely, we have previously demonstrated that a reduction in testing fluid osmolality increases disc height under a range of loading magnitudes (Vergroesen et al., 2016). This study confirms that osmosis is indeed important for the disc's biomechanical properties. Interestingly, the addition of PEG to the testing fluid particularly seemed to reduce the unloading recovery in a stable dose-dependent manner from day 1 (Figure 5a) whereas loading creep was progressively reduced with the loss of disc height (Figure 5b). Furthermore, we show that sufficient testing time is essential when evaluating fluid flow mechanics in order to reach a dynamic equilibrium. In equilibrium, the addition of PEG had reduced water content, disc height, and water inflow and outflow in a dose-dependent manner.

In the present study, the compressive stiffness of the discs was not affected by the change in osmotic gradient (Figure 6). Previously, stiffness has been associated with degeneration, but most changes were found with end-stage degenerative discs (Emanuel et al., 2015), where bone-to-bone contact may occur, which sharply increases the stiffness. Bezci et al. (2015) showed a moderate correlation between an increase in osmolality and the young's modulus (ρ=0.49-0.51) when using different saline solutions. The lack of an effect of PEG addition on compressive stiffness in the current experiment is possibly due to the measurement of stiffness in the relatively small ranges of 80-180N and 40-60N, as this might reduce accuracy of the stiffness measurement. Furthermore, stiffness measurements are difficult to compare between studies, as it is highly protocol dependent (Bezci et al., 2015). We performed our stiffness measurements on pre-stressed tissue at relatively higher strain rate, which may have attenuated the differences between experimental groups.

In contrast with the changes associated with degeneration, the time constants increased over the days in the 26 % PEG group (Figure 7c). Also the derivative of the disc height at the end of the recovery increased (Figure 7b). This means that the process of water in- and outflow is slower, presumably indicating that the hydraulic permeability of the intervertebral disc has been reduced. Possibly, the reduction in water content makes the extracellular matrix more compact, thereby decreasing the permeability (Perie et al., 2006). In contrast, the permeability of the matrix increases with disc degeneration (Iatridis et al., 1998). This is

reflected in shorter time constants with degeneration, and in discs being near equilibrium at the end of the day and night (Emanuel et al., 2015). The contrast between the results in the current study and those found with degeneration indicates that the changes of the extracellular matrix with degeneration do not only affect the osmotic attraction of water, but also the permeability to water. This is important, as both affect the fluid flow, and thereby the hydrostatic pressure in the disc. This hydrostatic pressure is known to stimulate the cells in the nucleus to produce proteoglycans (Ishihara et al., 1997). Loss of this pressure may thus reduce proteoglycan synthesis (Handa et al., 1997) and induce a positive feedback loop that stimulates a vicious cycle of degeneration (Vergroesen et al., 2015). The permeability within the disc is likely determined by the permeability of the nucleus pulposus, as the hydraulic permeability of the annulus fibrosus is an order of magnitude higher (Cortes et al., 2014), and is therefore likely not the limiting factor. Furthermore, the end-plate permeability presumably has limited effect on axial disc biomechanics, as total blockage of the endplate does not influence creep or time constants (Urban and Maroudas, 1981; van der Veen et al., 2007). Perie et al. (2006) found a negative association between nucleus permeability and proteoglycan content, which suggests that the loss of permeability and the loss of osmolality may both have the same aetiology. Further research on the effect of changes in disc tissue permeability on axial biomechanics should be conducted to further define the fundamentals of intervertebral disc biomechanics.

Although the goat is a quadruped animal, loads on caprine intervertebral disc are also predominantly in the axial direction (Smit, 2002). Degenerative interactions between the extracellular matrix and cells are not expected in this short time frame (Vergroesen et al., 2015). However, *in vitro* testing is an established method for assessing biomechanical behaviour in the intervertebral disc, especially if preconditioning is adequate (Wilke et al., 1998). The use of high-osmotic testing fluids does not reflect physiological conditions, but PEG addition has previously been used as a method of reducing the swelling pressure of nucleus explants *in vitro* (Urban and Maroudas, 1981; van Dijk et al., 2011), and others have altered saline content to increase or decrease testing fluid osmolality (Bezci et al., 2015; Vergroesen et al., 2016). As stated, the first minute of each phase was not considered for analysis as we were not interested in the immediate elastic response but rather in fluid flow. Considering the low permeability of the nucleus and the annulus (Cortes et al., 2014; Iatridis et al., 1998), fluid flow is a slow process. Furthermore, previous results

showed time constants due to osmotic fluid flow were in the order of hours (Vergroesen et al., 2016). Therefore, no significant amount of fluid flow was expected in the first minute. Due to effects of 12h storage between sacrifice and experimentation —a lack of loading and subsequent super-hydration— the first 8 hours of the experiment did not reflect a recovery phase as observed during the rest of the experiment. Therefore, the second night was considered the first unloading phase in the analysis. Nonetheless, it should be noted that the discs were already on PBS with added PEG. During the succeeding 5 days, disc behaviour in control discs was stable, indicating these days were adequate for analysis. All discs where subjected to the same forces, regardless of the area of the discs, as the exact cross sectional area is impossible to determine prior to testing. To reduce a possible effect of size, we used only two spines, and stratified for spine and disc levels. We feel that this was adequate since the number of discs per group is relatively small, but the effect of the PEG was very convincing, reflected in the low standard deviations and p-values for multiple parameters.

Altering the osmotic gradient between the intervertebral disc and surrounding fluids significantly alters axial biomechanics and mimics some features of degenerative biomechanical behaviour. Therefore, if regenerative therapies aim at restoring osmotic gradient in the disc we can expect an increase in water content, disc height and daily creep. However, it should be noted that the control caprine intervertebral discs were able to retain an average water content of 68% after multiple days of axial loading. Axial loading at these magnitudes generates pressures in excess of 1.0MPa in the nucleus of caprine intervertebral discs (Vergroesen et al., 2014), indicating the high osmotic pressure that can be generated by the disc's proteoglycans. Therefore, therapies aiming to restore intervertebral disc biomechanics by mimicking the nucleus pulposus should retain high water content under prolonged elevated pressures.

It can be concluded that the osmotic gradient generated by the disc's proteoglycans only explains a part of the biomechanical change with degeneration. The discs tested on fluids with added PEG lost water content, and the daily creep and recovery is reduced in a similar manner to degeneration. However, this experiment indicated the fluid exchange in experimental groups is much slower than in degenerated discs. This infers

that axial biomechanical behaviour of the intervertebral disc is determined by more than osmosis alone. A probable additional tissue property involved in intervertebral disc biomechanics is hydraulic permeability.

Conflict of interest statement

None of the authors report any conflicts of interest

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Figure 1: The simulated physiological loading regime, previously described by Paul et al. (Paul et al., 2012). For eight hours, low dynamic loading (40-60N) is applied as night time unloading, and during 16 hours, alternating blocks of 30 minutes of 40-60N and 80-180N are applied. All loading is applied as a sinusoidal wave with a frequency of 1Hz.

Figure 2: Biomechanical parameters of the loading study: **1** overall disc height loss, relative to the start of the experiment; **2** poro-elastic recovery during the unloading phase; **3** poro-elastic creep during the loading phase (N.B. the initial elastic displacement during the first minute is not included for 2 and 3); **4** (red line) rate of recovery during the first half hour of the unloading phase; **5** (red line) rate of recovery during last half hour of the unloading phase; **6** (black double arrow) disc stiffness at the end of the night (unloading phase); **7** (black double arrow) disc stiffness at the end of the day (loading phase).

Figure 3: a Water content for individual parts of the intervertebral disc for all groups; **b** Glycosaminoglycan content for individual parts of the intervertebral disc for all groups; **c** Hydroxyproline content for individual parts of the intervertebral disc for all groups. Full lines indicate main effects of group, dashed lines indicate post-hoc differences between groups, dotted lines indicate interactions between group and tissue. * $p<0.05$, # $p<0.0001$.

Figure 4: Typical examples of the displacement data over the days of the experiment. PEG groups show an increased overall loss of disc height over the experiment with increasing osmotic value of the culture medium.

Figure 5: Daily displacement, divided into: **a** unloading recovery; **b** loading creep; **c** recovery - creep. Full lines indicate main effects of day (on top of the figure) or group (to the right of the figure), dashed lines indicate post-hoc differences between groups, dotted lines indicate interactions between groups and days. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.0001$.

Figure 6: a stiffness values at the end of the unloading phase; **b** stiffness values at the end of the loading phase. Full line indicates main effect of day (on top of the figure) * $p<0.05$.

Figure 7: a The rate of recovery during the first half hour of night time over the days for all groups (start); **b** The rate of recovery during the last half hour of night time over the days for all groups (end); **c** Time constants for recovery over the days for all groups. Full lines indicate main effects of day (on top of the figure) or group (to the right of the figure), dashed lines indicate post-hoc differences between groups, dotted lines indicate interactions between groups and days. * $p<0.05$, ** $p<0.01$, # $p<0.0001$.