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## The poro-elastic behaviour of the intervertebral disc: A new perspective on diurnal fluid flow

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### ABSTRACT

Diurnal disc height changes, due to fluid in- and outflow, are in equilibrium while daytime spinal loading is twice as long as night time rest. A direction-dependent permeability of the endplates, favouring inflow over outflow, reportedly explains this; however, fluid flow through the annulus fibrosus should be considered. This study investigates the fluid flow of entire intervertebral discs. Caprine discs were preloaded in saline for 24 h under four levels of static load. Under sustained load, we modulated the disc's swelling pressure by exchanging saline for demineralised water (inflow) and back to saline (outflow), both for 24 h. We measured disc height creep and used stretched exponential models to determine time-constants. During inflow disc height increased in relation to applied load, and during outflow disc height decreased to preload levels. When comparing in- and outflow phases, there was no difference in creep, and time-constants were similar indicating no direction-dependent resistance to fluid flow in the entire intervertebral disc. Results provoked a new hypothesis for diurnal fluid flow: *in vitro* time-constants for loading are shorter than for unloading and *in vivo* daytime loading is twice as long as night time unloading, *i.e.* in diurnal loading the intervertebral disc is closer to loading equilibrium than to unloading equilibrium. Per definition, fluid flow is slower close to equilibrium than far from equilibrium; therefore, as diurnal loading occurs closer to loading equilibrium, fluid inflow during night time unloading can balance fluid outflow during daytime loading, despite a longer time-constant.

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### 1. Introduction

Diurnal loading of the human spine consists of ~16 h daytime loading, and ~8 h night time unloading, causing a 1.1% difference in body height between evening and morning (Reilly et al., 1984; Tyrrell et al., 1985). This is attributed to loss and gain of intervertebral disc height (McMillan et al., 1996; Reilly et al., 1984). Disc height changes due to axial compression and relaxation are the result of fluid flow (Adams et al., 1996; Iatridis et al., 1997; Koeller et al., 1984; McMillan et al., 1996; van Dieën et al., 2001; White and Panjabi, 1990), directed outward during loading, and inward during unloading (Urban and McMullin, 1988; Vergroesen et al.,

2014). Although there is superimposed short-term loading and unloading with changes in activities during the day; overall, posture change and long-term fluid flow follows a diurnal rhythm, and is balanced over time *i.e.* the disc is in dynamic equilibrium.

Although daily activity varies in intensity, spinal loading during daytime is generally higher than during night time rest (Wilke et al., 1999). Additionally, the period for loading is twice as long as night time rest; therefore, inflow must, on average, be faster than outflow in order to maintain equilibrium. Currently, the predominant hypothesis explaining this equilibrium is a direction-dependent resistance to fluid flow through the vertebral endplate, favouring fluid inflow over outflow (Ayotte et al., 2001). However, this hypothesis may be only part of the explanation, as it is based on the assumption that fluid flow—only—occurs through the vertebral endplate. This assumption is based on *in vivo* studies which show the majority of gadolinium and <sup>35</sup>S-sulphate to be transported through the endplate into the nucleus (Arun et al., 2009; Rajasekaran et al., 2004; Roberts et al., 1996; Urban et al.,

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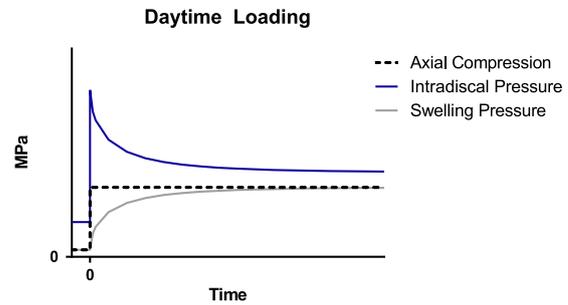
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1982). However, nutrient diffusion from blood vessels is something other than interstitial fluid flow (Nachemson et al., 1970; Urban et al., 1982); therefore, fluid flow via the annulus fibrosus should not be discounted. In fact, the hydraulic permeability of the annulus fibrosus is 10 times larger than that of the cartilaginous endplates (Cortes et al., 2013; Wu et al., 2014). Additionally, the annulus fibrosus is permeable along the entire circumference, but the endplates are only permeable in their centre (Nachemson et al., 1970); consequently, the permeable area for fluid flow is smaller for the endplates. Since the hydraulic permeability and the permeable area are larger, the annulus is a likely pathway for interstitial fluid flow; furthermore, this increased permeability and permeable area might overcome the larger distance between nucleus and annulus than between nucleus and endplate. Accordingly, *in vitro* studies have shown that sealing of the vertebral endplates did not influence the response of the intervertebral disc to loading or unloading (Schmidt et al., 2014; Van der Veen et al., 2007), which strongly suggests that interstitial fluid flow due to diurnal loading and unloading occurs through the annulus fibrosus rather than the endplates.

Experimentally, the fluid flow in an entire intervertebral disc is difficult to monitor; however, disc height changes which are in part dependent on fluid flow can be monitored. With diurnal loading or unloading the height of the intervertebral disc shows an instant visco-elastic response due to tissue deformation, followed by non-linear and time-dependent creep, due to poro-elastic fluid flow (Emanuel et al., 2015). Disc height creep can be described using mathematical models (Van der Veen et al., 2013). Previously, these models have shown time-constants for unloading to be 2.5–27 times longer for unloading than for loading (MacLean et al., 2007; O'Connell et al., 2011b), indicating a slower fluid inflow than outflow, in contrast to *in vivo* loading (Reilly et al., 1984; Tyrrell et al., 1985). The paradox that—*in vitro*, unloading creep has a longer time-constant than loading creep, while *in vivo*, overnight fluid inflow is faster—has thus far not been resolved.

In understanding diurnal fluid flow it is important to consider that although they are balanced, the driving forces for out- and inflow of fluid are different in character: during loading, the mechanical compressive pressure increases intradiscal pressure, which expels water from the intervertebral disc (Ayotte et al., 2001; Vergroesen et al., 2014); whereas, upon unloading the swelling pressure of the nucleus pulposus attracts water into the disc (Urban and McMullin, 1988). This swelling pressure is generated by the proteoglycans' ability to attract and bind water, and is defined as: “equal to the compressive pressure that needs to be applied for the tissue to maintain its state of hydration” (Urban and McMullin, 1988). Swelling pressure (or potential) changes with nucleus hydration, and with alterations to the balance in the osmotic charge between the intervertebral disc and the surrounding tissues (Urban and McMullin, 1988, 1985; Urban and Maroudas, 1981). This results in the following process: during loading, intradiscal pressure increases, which expels water from the disc, and disc height is lost (Vergroesen et al., 2014). Simultaneously, with lower hydration swelling pressure increases exponentially (Urban and McMullin, 1988, 1985; Urban and Maroudas, 1981), the increase in swelling pressure opposes the effect of loading until both are balanced, and fluid flow stops (Fig. 1). Inversely, during unloading, the swelling pressure attracts fluid into the disc, increasing disc height; but as the disc rehydrates, the swelling pressure decreases and intradiscal pressure rises. Presumably, the pressure difference between intradiscal pressure and swelling pressure determines the rate of fluid in- or outflow. *In vivo*, this interplay between swelling pressure and intradiscal pressure does not reach equilibrium, because fluid flow is slow due to low permeability of the tissues (Cortes et al., 2013).



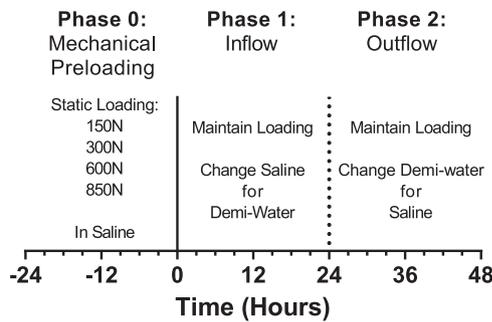
**Fig. 1.** The relation between mechanical loading of the spine and intradiscal and swelling pressures. Getting out of bed at  $T=0$  instantly increases intradiscal pressure at a level of 1.5–2 times the axial pressure (dashed black line) (Nachemson, 1981). This increase in intradiscal pressure expels water from the intervertebral disc, but with this reduction of water content, the intradiscal pressure decreases (dark blue line) (Van der Veen et al., 2005; Vergroesen et al., 2014). The reduction in water content in turn increases the swelling pressure within the intervertebral disc (light grey line) (Urban and McMullin, 1988, 1985; Urban and Maroudas, 1981). The difference in stresses at equilibrium is due to a strain in the annulus fibrosus (intradiscal pressure is  $\sim 0.1$  MPa in unloaded conditions). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

By investigating disc height creep, the fluid flow can be investigated *in vitro* (Van der Veen et al., 2005); however, there are some considerations that need to be accounted for if creep is to be translated to fluid flow alone. Firstly, a separation of visco-elasticity (tissue deformation) and poro-elasticity (fluid flow) should be made (Emanuel et al., 2015), which also separates the creep within the attached bone and endplates from the creep within the disc (Van der Veen et al., 2008). Secondly, loading magnitude is important as disc height and intradiscal pressure depend on loading history of the disc (Vergroesen et al., 2014; Wilke et al., 1999), and hydraulic permeability of tissues could be affected by the strain in the annulus (O'Connell et al., 2011a) or bulge of the annulus fibrosus (Brinckmann and Grootenboer, 1991). Thirdly, the duration of the measurement is important as modelling accuracy is increased with longer measurements (Van der Veen et al., 2013). Finally, the disc should be in equilibrium prior to the experiment to correct for hydration status (O'Connell et al., 2011b).

Here we investigate the fluid in- and outflow in an entire intervertebral disc by monitoring disc height changes *in vitro*. In order to separate the visco- and poro-elastic effects of different loads, we start by applying mechanical pressure to a disc submerged in saline until disc height creep stops, then, intradiscal pressure, swelling pressure, tissue strain and disc bulge are in equilibrium (phase 0: mechanical preloading). After reaching equilibrium, we change saline to demineralized water to increase the swelling pressure of the disc (phase 1; inflow). This should induce fluid inflow into the disc (*i.e.* disc height increase), by keeping the mechanical load constant we expect limited visco-elastic tissue deformation. Subsequently, changing back to saline should induce fluid outflow, restoring disc height (phase 2: outflow). We investigate the disc height creep curves with a stretched exponential function. Using the results of this study, we were able to formulate a new hypothesis on the balance of diurnal fluid flow.

## 2. Materials and methods

Five lumbar spines were harvested from 3 to 5 year old skeletally mature female Dutch milk goats, and stored at  $-20$  °C. While frozen, the discs (L1–L2 to L4–L5) were located using fluoroscopy, and adjacent vertebral bodies were removed  $\sim 3.5$  mm from the disc with a band saw. Subsequently, the cutting edge was brushed clean to remove sawing debris, and rinsed to remove blood clots.



**Fig. 2.** Experimental loading protocol of the intervertebral discs. The total testing duration is 72 h, divided into three phases of 24 h. During phase 0, a static load is applied as a preload; during phase 1, the static load is maintained, but medium is changed from saline to demi-water; during phase 2, static load will be continued but medium is restored to saline.

During storage, discs were wrapped in saline soaked gauzes. Before testing, discs were thawed overnight in a saline bath to allow rehydration.

Intervertebral discs were tested at 37 °C. Porous platters at both endplates ensured fluid exchange via both the endplates and the annulus. Each disc was tested for 72 h, divided into three phases (Fig. 2): 24 h of mechanical preloading until equilibrium (phase 0), and two 24-h phases for inflow and outflow (phases 1 and 2, respectively). During the first and last 24 h of the test, discs were immersed in 0.9% saline (~295 mOsmol). During the second 24 h (phase 1), saline was exchanged for demineralised (~0 mOsmol), in order to temporarily increase swelling pressure of the disc. When changing the fluid, the system was flushed in order to remove all the fluid of the previous phase.

In order to account for the effect of annulus strain on tissue permeability, four different static loading levels were compared (150 N, 300 N, 600 N and 850 N). No data is available to directly transfer this to *in vivo* caprine intradiscal pressures, however, when we consider a linear response of caprine intradiscal pressure to load (Vergroesen et al., 2014), loads correspond to pressures of approximately 1.1 MPa, 2.2 MPa, 4.3 MPa, and 6.0 MPa, which we can combine with *in vivo* ovine data (Reitmaier et al., 2013) and from an interbody spinal cage in a goat (Doormans et al., 2004). These would predict the loads to correspond to relaxed standing, and different activities such as lying down, standing up, and jumping on a haystack, respectively. In humans, these pressures would correspond to relaxed standing, bending forward, carrying 20 kg with a straight back, and picking 20 kg up from the floor, respectively (Wilke et al., 1999). Load was applied axially with a hydraulic mechanical testing device (Instron 8872 Canton, Massachusetts). Load and displacement were registered at 2 Hz. One disc per spine was tested per load level to correct for inter-animal variability.

In cartilaginous tissues, osmotic and mechanical loading are considered to generate equal stress on the tissues (Lai et al., 1998); therefore, reducing medium osmolality reduces stress from mechanical loading, but without instant viscoelastic tissue deformation. Stresses can be calculated using the Van't Hoff equation (Hoff, 1887), which states:

$$\text{Osmotic Pressure (MPa)} = 0.1 \cdot I \cdot C \cdot R \cdot T.$$

$I$  is the dimensionless dissociation constant of the solute,  $C$  is the molarity of the solution,  $R$  is the gas constant, and  $T$  is the temperature at testing. For this experiment,  $I=2$  for NaCl;  $C=0.1538$  osmol for 0.9% NaCl;  $R=8.21 \times 10^{-2}$  L atm K<sup>-1</sup> mol<sup>-1</sup>; and  $T=310$  K. This results in osmotic stress reduction of 0.78 MPa between saline and demineralized water. Previously, we have shown that in goat intervertebral discs intradiscal pressures of approximately 0.78 MPa are generated with axial loads of 99 N (Vergroesen et al., 2014); therefore, we presume a reduction in axial loading of 99 N during Phase 1.

Disc height creep can be described with a stretched exponential or Kohlrausch-Williams-Watts function (KWW) (Van der Veen et al., 2013). The KWW stretched exponential is of the form:

$$x(t) = d_{\infty} + (d_0 - d_{\infty})(e^{-(t/\tau)^{\beta}})$$

where  $t$  is the elapsed time,  $\tau$  the time-constant,  $\beta$  the stretch parameter,  $d_0$  the disc height at  $t=0$  and  $d_{\infty}$  the disc height at equilibrium (the asymptote when  $t$  is infinite). The fitted curves were optimised for the least summed square of the difference between the fitted curve and the measured data, and  $R$ -squared was used to describe the quality of the fit. The changes in  $d_{\infty}$  between phases are expressed as  $\Delta d_{\infty}$ .

Because compressive stiffness is a biomechanical parameter that is often used to assess disc function in axial loading studies (Paul et al., 2013), this was additionally investigated and discussed in Supplement #1.

## 2.1. Statistical analyses

Using SPSS (IBM Software, Armonk NY, USA, version 20 for Windows), Huynh-Feldt repeated measures analyses of variance were performed on the dependent variables:  $d_{\infty}$ ,  $\tau$ , and  $\beta$  to test the effects of load phase (within factor, 3 levels: mechanical preloading, inflow and outflow) and to test the effect of loading level (between factor, 4 levels: 150 N, 300 N, 600 N or 850 N). Repeated contrasts were used to determine differences between phases. If interactions were found, an ANOVA for the evaluation of the effect of load level within a single phase was used. Finally, quadratic fits over  $\Delta d_{\infty}$  data for phase 1 and 2 were made in Graphpad Prism (Graphpad software, La Jolla, CA, USA, version 6.04 for Windows).

## 3. Results

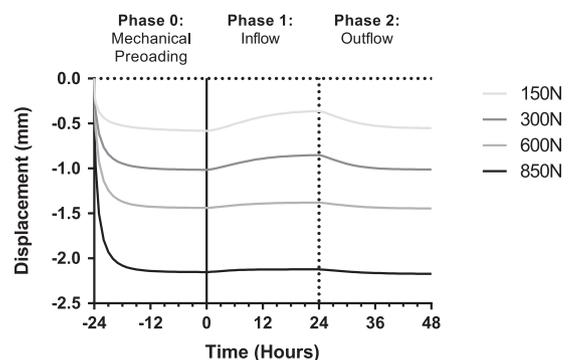
### 3.1. The reduction of medium osmolality increased disc height

During preloading, the height of all intervertebral discs decreased in response to the applied mechanical load (Fig. 3). During phase 1, saline was exchanged for demineralised water, and all discs gained height. In phase 2, the disc height that was gained during phase 1 was lost again.

The KWW model generally fitted well to the data ( $R^2$  values > 0.95). Four discs however fitted less well and their creep curves were subjected to additional visual inspection. Generally, only the fit for one single phase was not good; therefore, data of that phase were then excluded for: one disc of the 150 N group during phase 0, one disc for the 600 N group for phase 0; and one disc of the 850 N group did fit well upon visual inspection, but due to some noise in the displacement signal had a lower  $R^2$  (0.76). Therefore this disc was not excluded. An extended table of the discs used and the numerical results of fits for all phases is included in Supplement #2.

### 3.2. Disc height changes are related to applied loads

There was a significant effect of load level for disc height at equilibrium (Fig. 4,  $p=0.004$ ). In phase 0, the asymptote of the creep ( $d_{\infty}$ ) was lower for higher load levels ranging from -0.6 to -2.15 mm, approximately 15% and 54% loss of original disc height (~4 mm) for 150 N and 850 N, respectively. For all load levels, there was a significant difference in disc height between phase 1 and 0 ( $p < 0.05$ ), and phase 1 and 2 ( $p < 0.05$ ), but no significant difference between  $d_{\infty}$  in phase 0 and 2 ( $p=0.376$ ). During inflow and outflow, the difference between the asymptotes ( $\Delta d_{\infty}$ ) was larger with lower load levels ( $p < 0.0001$ ), and this relationship was exponential (Fig. 5,  $R^2=0.81$  and 0.92, respectively). The



**Fig. 3.** Typical examples of disc height changes over all three phases of the experiment, for discs from all experimental groups. During mechanical preloading we see a reduction in disc height relative to the applied load. During phase 1, we see an increase in disc height (fluid inflow) due to the decrease of medium osmolality. During phase 2, medium osmolality is restored, and disc height is reduced towards preload levels.

absolute change in disc height from phase 0 to phase 1 was comparable to the change in disc height from phase 1 to phase 2 ( $p=0.376$ ), indicating the same volume of fluid in- and outflow.

### 3.3. Time-constants for inflow are decreased with higher load levels

There was an interaction between load level and phase (Fig. 6,  $p < 0.0001$ ): during preload and outflow, there was no effect of load level ( $p=0.338$  and  $p=0.557$ , respectively), but during inflow, time-constants decreased with increasing load ( $p < 0.013$ ). Overall, there was an effect of phase ( $p < 0.0001$ ): the time-constant for mechanical preloading was an order of magnitude smaller than the time-constants for inflow and outflow ( $p$ -values  $< 0.0001$ ). There was no clear change in time-constant between inflow and outflow (interaction  $p < 0.0001$ ), time-constants for outflow were reduced at low load levels (150 N  $p=0.03$ , 300 N  $p=0.001$ ), but remained similar to inflow values at higher load levels (600 N  $p=0.318$ , 850 N  $p=0.064$ ).

### 3.4. The shape of the curve was not affected by load level or direction of fluid flow

There was no effect of load level on coefficient beta of the KWW model ( $p=0.864$ ). However, beta for preloading ( $0.570 \pm 0.086$ ) was significantly lower than beta for inflow and outflow ( $p < 0.0001$ ,  $1.46 \pm 0.25$  and  $1.33 \pm 0.20$ , respectively). There was no difference in beta between inflow and outflow

( $p=0.244$ ), nor was there an interaction effect between load and phase ( $p=0.931$ ).

For one disc of the 850 N group, the test was repeated after 48 h at 150 N. Disc behaviour was consistent with the other 150 N tests, indicating that no damage had occurred.

## 4. Discussion

This study investigated the differences between fluid in- and outflow in an intact intervertebral disc under different levels of mechanical load. As expected, preloading reduced disc height in a dose-dependent manner. The increase of swelling pressure due to lowering of medium osmolality induced fluid flow into the disc and partially restored disc height, with more disc height restored at lower loading levels. Upon restoration to 0.9% saline, disc height was restored to the disc height after preloading.

In this experiment, fluid volume for in- and outflow was not significantly different. The variables  $\Delta d_{\infty}$  and  $\beta$ , did not differ significantly, and only for low load levels tau was reduced for outflow compared to inflow. This implies that: (1) a difference in flow direction cannot explain the large differences in time-constants for *in vitro* loading and unloading in an entire intervertebral disc; and (2) the direction-dependent flow through the endplate (Ayotte et al., 2001) has no significant influence in directing fluid flow in the entire intervertebral disc. However, a small effect of direction dependent tissue permeability cannot be

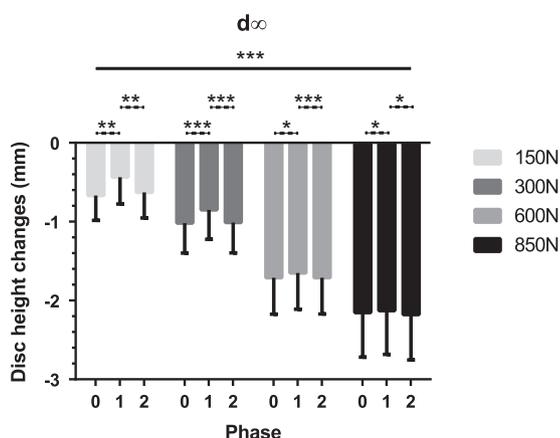


Fig. 4.  $d_{\infty}$  for all phases and all loading conditions. Mean and SD values are given. In phase 0,  $d_{\infty}$  is reduced with increased load. During phase 1, disc height is partially restored, and in phase 2, previously gained disc height is lost towards preload levels.

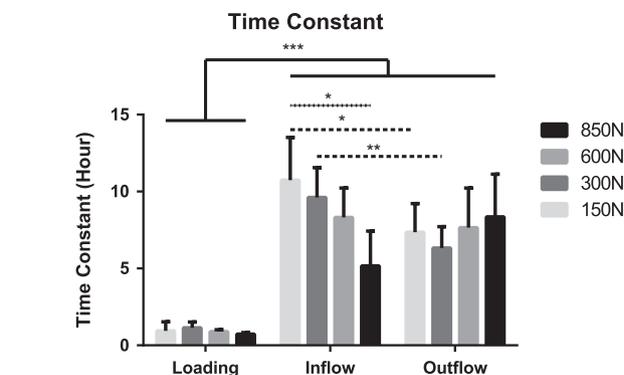
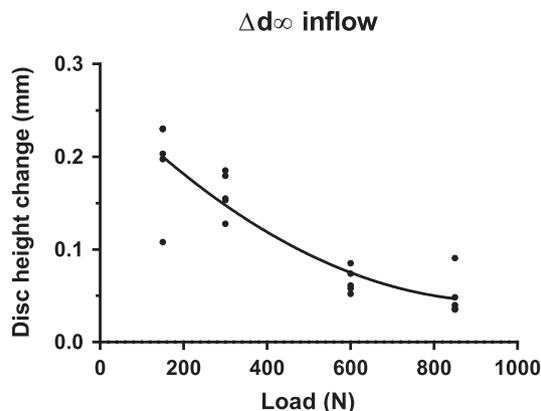


Fig. 6. Time constants for all phases and all loading conditions. Mean and SD values are given. Mechanically induced fluid flow had much shorter time constants than osmotic in or outflow (full line -  $p < 0.0001$ ). Within phase 1, there was a decrease in time constant with increasing load (dotted line -  $p < 0.013$ ). Time constants for 150 N and 300 N groups significantly decreased from phase 1 to phase 2 (dashed lines  $p=0.03$  and  $p=0.001$ , respectively), but there was no effect in the 600 N and 850 N group.

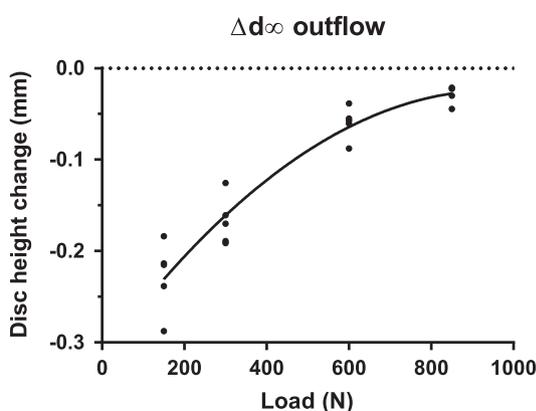


Fig. 5. The changes in disc height ( $\Delta d_{\infty}$ ) for each individual disc upon inflow and outflow, the relationship between load and changes in disc height was well described with exponential fits ( $R^2=0.81$  for inflow, and  $0.92$  for outflow).

disregarded, as time-constants decreased with increasing load in inflow, but this effect was inverted for low loads during outflow. However, this is possibly due to the discs not being fully in equilibrium at the end of phase 1: 24 h testing is marginally sufficient with a time-constant of 8–10 h, and some creep still occurs in the 150 N and 300 N discs (Fig. 3). Else, this might indicate that annulus strain or endplate deformation has a direction-dependent effect on fluid flow: it hampers inflow at low load levels, and enhances outflow at low load levels. Overall, tissue strain presumably remained similar during the entire experiment as compressive stiffness was not changed during phases 1 and 2 (Supplement #1).

If diurnal fluid flow is not primarily balanced through direction-dependent tissue permeability, *in vivo* a likely explanation may lie in the difference in character between mechanically induced fluid outflow, and swelling pressure induced fluid inflow. We found an order of magnitude difference between the time-constant of the preloading phase and both osmotic phases (Fig. 6). This concurs with previous results as time-constants were 2.5–27 times longer for swelling pressure induced unloading creep than for mechanically induced loading creep (MacLean et al., 2007; O'Connell et al., 2011b). However, contrary to the hypothesis of Lai et al. (1998), current results also imply that osmotic loading is not wholly equivalent to mechanical loading: there was a large difference in time-constants between the preload and outflow phase, and beta values for osmotic loading and unloading were significantly different from the mechanical loading phase. Mechanical loading might stress the entire tissue instantaneously whereas osmotic loading stress might be hampered by the low permeability of the tissues. Additionally, we hypothesise that mechanical loading can generate a larger pressure difference between the intradiscal pressure and the swelling pressure (up to 6.0 MPa in this study), compared to the potential of osmotic pressure (0.78 MPa in this study). This large difference in pressure leads to a faster initial fluid flow, hence a shorter time-constant. This is reflected by the results of the time-constant for inflow, with increasing load levels (*i.e.* higher swelling pressure) time-constants are reduced (Fig. 6).

Axial mechanical loading reduced  $d_{\infty}$  in a dose-dependent manner. This is expected to occur in a non-linear manner, as swelling pressure increases exponentially with reduced hydration (Urban and McMullin, 1988, 1985; Urban and Maroudas, 1981). This was confirmed for both inflow and outflow (Fig. 5). Therefore, using the data from phases 1 and 2, we confirm that a specific external load will have a specific  $d_{\infty}$ , and that the relationship between load and  $d_{\infty}$  is non-linear.

Based on the results of this study we provide the following hypothesis for the balance of diurnal fluid flow: during normal ambulant behaviour, daytime loading, and night time unloading both have a specific  $d_{\infty}$ . Because the time-constant for loading is shorter than for unloading, in the diurnal rhythm: the loading  $d_{\infty}$  is more closely approached than the night time unloading  $d_{\infty}$ . This proximity to the loading asymptote can be seen by the flattening of the daytime curve in the diurnal changes in posture (Reilly et al., 1984; Tyrrell et al., 1985). That the unloading  $d_{\infty}$  is hardly approached in the diurnal rhythm can be observed upon prolonged unloading during bed-rest and spaceflight: body height can increase up to 5 cm (Belavý et al., 2011a, 2011b; Cao et al., 2005; Urban and Maroudas, 1981; Wing et al., 1991) or 300% of normal diurnal disc height changes (LeBlanc et al., 1994; Reilly et al., 1984; Tyrrell et al., 1985). Because fluid flow is non-linear and time dependent, the distance towards the asymptote at  $d_{\infty}$  also determines the rate of fluid flow, rather than the time-constant alone. This explains the paradox that although the time-constant for loading is faster and the time for unloading is

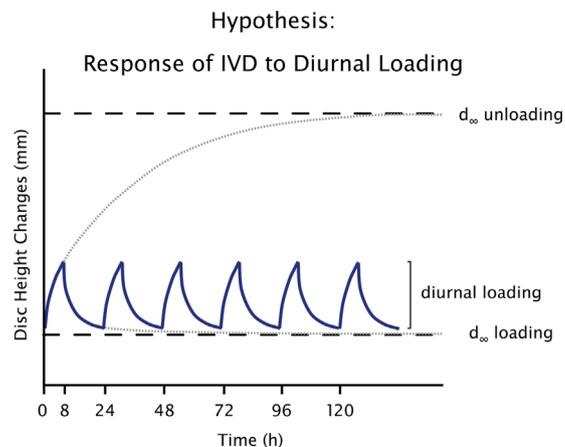


Fig. 7. Hypothesis of the response of the IVD to diurnal loading (blue line), 6 days of night time (8 h) and daytime (16 h) are shown. In a normal diurnal rhythm, loading time constants are shorter than unloading time constants, and the  $d_{\infty}$  of loading (dashed black line in the bottom) is approximated closer than the  $d_{\infty}$  of unloading (dashed black line on top). With an increase of the distance to the asymptote  $d_{\infty}$ , the fluid flow is increased. Dotted grey lines indicate hypothetical disc height changes if the time of loading or unloading would be extended.

shorter, the total diurnal fluid flow is equal for unloading and loading (Fig. 7).

The use of goat intervertebral discs to model human intervertebral discs is adequate, as stresses on the intervertebral discs of both humans and quadruped animals are predominantly in the axial direction (Smit, 2002; Smit et al., 1997). The KWW model fitted to the creep curves quite well; however, simultaneous use of both the KWW model and a Double Voight model (DV) has been proposed in the literature (Van der Veen et al., 2013). Therefore in Supplement #2, we also fitted a DV model to the data, and found similar trends, indicating our KWW results are valid.

There are some limitations to the current study. In hindsight, we should have preloaded all discs at low loads until equilibrium, prior to a mechanical loading phase to investigate the change in  $d_{\infty}$  with increased mechanical compression. Additionally, at the end of the experiment, we should have included a mechanical unloading phase, towards the preload level, to evaluate differences between mechanical and osmotic loading and unloading. The absence of additional preloading would have affected the results of the current mechanical preloading phase, but not of the inflow and outflow phases, which were performed from equilibrium. The absence of mechanical unloading does not allow us to further substantiate our hypothesis with data showing a faster recovery with higher loading levels. However, including the proposed phases would probably not have affected our hypothesis for diurnal fluid flow.

There are some implications when the determinants of diurnal fluid flow are indeed adequately described by the current hypothesis. Firstly, diurnal fluid flow in the intervertebral disc is dependent on swelling pressure of the disc (*i.e.* the proteoglycan content of the nucleus and annulus), rather than endplate function. To compare current caprine results to human discs: caprine GAG content of nucleus and annulus is approximately 370 and 78  $\mu\text{g}$  GAG/mg dry weight, respectively (Paul et al., 2012), which is comparable to humans of 40–60 years of age (Antoniou et al., 1996). Secondly, a reduction of proteoglycans with degeneration would shift both loading and unloading  $d_{\infty}$  downwards. Thirdly, the bandwidth between loading and unloading  $d_{\infty}$  will decrease because of the non-linear dependency of swelling pressure on proteoglycan content (Urban and McMullin, 1988). These effects could be seen experimentally as well as clinically as an overall reduction in disc height, and a reduction in the amplitude of daily

disc height changes. These swelling-pressure-dependent outcome measures are probably more sensitive in biomechanically determining disc degeneration than the often used axial compressive stiffness (Supplement #1).

## 5. Conclusion

This study explored fluid flow into and from intact intervertebral disc under different levels of mechanical load. Reduction of culture medium osmolality induced an increase in disc height, and the subsequent restoration induced a decrease in disc height, indicating fluid in- and outflow. Creep varied with the mechanical load applied. There was no difference in volume of disc height change and time-constants were similar between in- and outflow phases indicating no direction-dependent resistance to fluid flow in the entire intervertebral disc. Results provoked a new hypothesis for diurnal fluid flow: the time-constant does not describe the actual rate of fluid flow; close to equilibrium fluid flow is slower than far from equilibrium. *In vitro* time-constants for loading are shorter than for unloading and *in vivo* daytime loading is twice as long as unloading, thus the intervertebral disc is closer to loading equilibrium than to unloading equilibrium. Thereby, fluid inflow is faster during the night than fluid outflow during the day.

## Conflict of interest statement

None of the authors has a conflict of interest regarding this work.

## Author contributions

Pieter-Paul A. Vergroesen\* Analysis and interpretation of the data, drafting of the manuscript, critical revision of the manuscript, final approval.

Albert J. van der Veen\* Research design, acquisition, analysis and interpretation of data, critical revision of the manuscript, final approval.

Kaj S. Emanuel Interpretation of the data, critical revision of the manuscript, final approval.

Jaap H. van Dieën Analysis and interpretation of the data, critical revision of the manuscript, final approval.

Theodoor H. Smit Research design, interpretation of the data, critical revision of the manuscript, final approval.

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All authors have read and approved the final version of this manuscript.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jbiomech.2015.11.041>.

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