Intervertebral disc degeneration
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Chapter 1.

General Introduction

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Low-back pain and intervertebral disc degeneration

Low-back pain (LBP) is the most common medical complaint in Western society (1), encompassing immense ensuing socio-economic costs (2-4). It is widely recognized that multiple factors underlie the complex and broad spectrum of pathologies linked to LBP (5-7). When regarding somatic causes, intervertebral disc (IVD) degeneration, or degenerative disc disease (DDD), is strongly associated with both the presence and severity of LBP complaints (8-11). Multiple large general population-based studies in the last decade have provided strong evidence for their correlation (12-15). Presently, there are no curative therapies for patients with symptomatic DDD. Treatment strategies are directed at symptom relieve and comprise exercise programs and physical therapy (16), pain medication (17) and acupuncture (18-23). For patients with severe, and severely symptomatic DDD, surgical salvage procedures are the only option. These involve removal of the disc followed by fusion or arthroplasty of the motion segment, which results in only moderate outcomes (24-27).

IVD anatomy and function

The intervertebral disc is the central part of the spinal motion segment. The disc functions to permit limited motion and flexibility of the spine, while maintaining segmental stability and absorbing and distributing external loads on the vertebral column. Its structure is complex and consists of several tissue types. The IVD is the largest avascular structure in the body and is subjected to substantial loading conditions (28). The central structure of the disk, the nucleus pulposus (NP), is a gel-like substance comprising predominately of collagen type 2 and proteoglycans (PG’s), mostly glycosaminoglycans (GAGs). Radially confining the NP is a highly-organized sheets of fibrils arrangement in laminae, which is called the annulus fibrosis (AF) (29). The laminae, usually around 20 to 25, are composed mainly of collagen type 1 fibrils and elastin, ideal to resist tensile forces (30;31). The IVD is one of the most sparsely cellular tissues in the body, with cell densities at maturity around $4 \times 10^6$ cells/cm$^3$ in the NP and $9 \times 10^6$ cells/cm$^3$ in the AF. The role of the chondrocyte-like and fibroblast-like cells residing in the NP and AF matrix is not completely understood; whether they serve an active role to maintain extracellular
matrix (ECM) content or are the passive remnants or senescent cells of their embryologic predecessors, (the notochordal and) mesenchymal cells, is still subject of research and debate (28;32-34). Covering the IVD proximally and distally are the cartilage (CEPs) and vertebral (VEPs) endplates (35). The CEP is a layer of hyaline-like cartilage that is thought to be responsible for most of the nutrients exchange with the adjacent vertebral body (VB). One IVD and two VB constitute the motion segment (MS), which is the functional unit of the spine. The motion-segment has shock absorbing properties mainly due to the high intradiscal pressure (36-39). The osmotic pressure is build up by the GAGs ability to retain or imbibe water and is both constrained by the annulus and enabled by the AF’s porous structure allowing fluid-exchange. The viscous-osmotic features of the NP, together with the porous-elastic characteristics of the AF give the healthy IVD its unique poro-elastic properties (40;41). These poro-elastic properties give the IVD a direct stiffness at an initial axial load and extra recuperating ability afterwards, making it capable of coping with peak loads as well as longer lasting mechanical burdens (42-44).

**Current concept of DDD etiology and the role of mechanical loading**

Intervertebral disc degeneration is considered to be a multifactorial process (45), in the physiological sense euphemistically referred to as “natural ageing of the spine” (46-50). Many vastly different risk factors have been identified that may aggravate this “natural process”, such as genetic predispositions (51-57), trauma (58-60) and infections (61-63) of the spine, loss of nutrient supply (64-66) to the disc due to atherosclerosis and stenosis of lumbar arteries (67-69), cardiovascular disease (70;71), high cholesterol (72), obesity (73) and diabetes (67;74). The degenerative cascade of the IVD starts in the nucleus pulposus (NP) where numerous incentives can cause a loss of proteoglycans (PGs, mostly glycosaminoglycans; GAGs) from the extracellular matrix (ECM), causing a decrease in the discs’ capability to retain or imbibe water (75). This affects its biomechanical function (i.e. poro-elastic behavior), as it hampers the disc’s ability to recover from daily loading conditions. Subsequently, creep (e.g. irreversible deformation) with loss in disc height and other gross morphological changes will occur over time (76;77).
Mechanical loading is considered to be the major extrinsic cause of intervertebral disc degeneration (78-80). The mechanism by which loading causes IVD degeneration (e.g. instigates loss of PGs) is subject of debate and somewhat paradoxical when considering that load bearing is the primary function of the IVD (81;82). Intervertebral discs are continuously under considerable pressure even during rest. Moreover, mechanical loading is known to be a natural stimulus to chondrocytes and regarded to be essential for maintenance of the homeostasis in cartilaginous tissue by facilitating fluid flow and distribution of nutrients towards and waste products from the cells (78;83;84). Furthermore, it has been reported that threshold values for beneficial or detrimental effects of static and dynamic loading differ between disc regions (nucleus, inner- and outer-annulus) (85-93).

**Disc region specific response to loading; a link to lumbar herniation**

Lumbar disc protrusion and herniation are age-related phenomena, that coincide with degenerative disc disease (DDD) (94-102). The potential connection is unclear, as a recent article by Lama et al. underlines (103). In their clinical study on human herniated discs of working age adults, it was shown that herniated discs needing surgery had only mild to moderate degeneration on the Pfirrmann score, and the herniated nucleus pulposus (NP) tissue did not show signs of degeneration (no significant loss of proteoglycans or water compared to controls). Herniations of the disc occur more frequently in the lower lumbar spine with a predilection of the annulus tear in the posterolateral corner (104). Spinal loading conditions have been identified to be major risk factors for developing a lumbar hernia (105-108). In fact, recent observational studies in the general adult population found that physical loading (109-111) and sitting hours (a static axial load on the spine) (112) are the most important extrinsic risk factors for developing a lumbar hernia. However, it is still unclear why some people develop (symptomatic) degenerative disc disease and debilitating hernias, while others have “uncomplicated” ageing and degeneration of their lumbar IVDs (94;113;114). Studies on human and various types of animal (lumbar) IVDs have shown a correlation between high mechanical forces applied to the disc, and degenerative changes (89;115-119). Both high static and high dynamic
axial overloading have negative effects on disc cells and matrix, but effects can vary by region, both nucleus and annulus, as well as anterior versus posterior region (87;117;120-127).

All factors considered, mechanical loading is a factor of interest, as it is one of the few factors that can be manipulated. However, we need to better understand its protective and harmful effects on the IVD. Mechanical loading conditions might be a determining factor whether lumbar discs age naturally, degenerate or herniate. If so, some regions of the IVD might be more prone to degenerate due to a certain overloading condition than others.

**Recognizing early intervertebral disc degeneration**

In order to therapeutically act on IVD degeneration, we need to understand its etiology better; how does the mechanical environment interact with the IVDs cell and matrix and can overloading cause a loss of GAGs from the NP, that starts the degenerative cascade towards DDD? How does early intervertebral disc degeneration present when looking at the biomechanical properties of the disc? And how can we better visualize and quantify the changes in the IVD involved with early stage degeneration. If we can identify the first degenerative changes, preferably before the disc has suffered irreversible damage, we could intervene to potentially slow down or halt the detrimental effects. The functional changes in the IVDs biomechanics that lead to irreversible height loss are poorly understood. And pain as well as IVD mechanical behavior cannot be seen on a still image (128). In order to identify early DDD, we need to find ways to more reliably measure changes involved with its onset. Novel parameters should be tested for their discriminative capabilities to recognize early and region-specific degeneration (129;130).

Focusing on how the disc behaves during loading and recovery, could prove more meaningful in early identification of DDD than assessing relative height loss alone. The biomechanical behavior of the IVD has been characterized in experimental settings with parameters such as stiffness and relaxation time-constant (131-133). However, the matrix’ poro-elastic properties give the IVD a non-linear subsidence and recovery behavior, which results in a large variation in calculated material stiffness, depending on the moment chosen to assess them (134;135). The possibilities to characterize disc behavior in a quantitative manner using modeling
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with stretch exponential function fitting, which is less sensitive to timing, has been studied previously (38;44;136;137). However, the fit parameters have not yet been tested for their ability to distinguish the biomechanical changes occurring with loss of GAGs, as the primary phase of disc degeneration.

T2-weighted appearance (signal intensity) of the disc is the resultant of the number of protons (mostly from water) in the nucleus and annulus fibrosis. T2-relaxation time based MR techniques cannot provide information on the nature of the water loss or gain in the disc (138;139). Whether the water loss is due to daily activity and diurnal rhythm (circadian; which is known to influence both water and height parameters) (140-145), or a water content drop in the ECM due to a loss of PGs from the matrix and therewith the (in)ability to retain water cannot be distinguished (146-148). T2 images is thus an indirect measure of potential matrix degeneration and can be obscured by the influx of fluids during an inflammatory process, by the presence of free water in the lacunas of degraded ECM (149) or even more systemic aberrancies in the motion segments blood flow (67;69). Also, when clinically significant height and T2 signal loss of affected disc levels have occurred, the degenerative cascade will already have reached a stage in which matrix integrity has been lost to a degree that IVD mechanics is failing permanently and the IVD is deemed unreparable with anti-inflammatory or regenerative modalities (150). Novel quantitative MR techniques such as T1rho (T1r) and apparent diffusion coefficient (ADC) have been suggested to be more accurate in detecting true matrix changes in cartilaginous tissue such as the IVD (151;152). However, these parameters have yet to be directly compared to gold standard quantitative T2 mapping, histological degeneration grading and biomechanical function parameters for IVD degeneration in their ability to distinguish early DDD better and in a region-specific manner (149;153).

Approaches to study the IVD

There is a long and continued effort to develop preventive or curative therapies for symptomatic DDD. Efforts are hampered by the complex multifactorial nature of both LBP and DDD (154), as well as a more basal lack of understanding of the mechanisms behind degenerative disease of cartilaginous structures such as the IVD (155-157). As our fundamental knowledge on the etiology expands, therapeutic
targets can be identified and new treatment strategies developed to potentially slow down or halt the progression of DDD (158-162). Mechanical loading has been extensively studied and evidence has gathered that it is a major extrinsic factor in preservation of disc homeostasis on the one hand and the onset of degeneration of the IVD on the other hand (83). More detailed knowledge is needed on exactly how mechanical conditions influence the disc (163). Various experimental models are utilized to get answers to specific questions on the role of particular factors involved in DDD. Human cadaveric spines and IVDs would theoretically be the ideal specimens for such investigations. However, this material is only available in small quantities and are never fresh (more than 8 hours post-mortem strongly decreases IVD (cell) viability) due to legislation. Moreover, human cadaveric material is mostly from old individuals, who usually have severely degenerated spines. This rules these specimens out for studies on the etiology of IVD degeneration. Post-mortem cooling or even freezing and the use of fixation techniques (i.e. formaldehyde) render specimens unusable for any type of experimental application, such as mechanical or (cell) biological (164-169). It is very difficult to study the effects of loading in in vivo (animal) models, because they lack direct control and monitoring of mechanical conditions of the IVD. In addition, in vivo methods are costly and raise ethical concerns (170-172). In vitro cell culture models are less appropriate because these cannot mimic the specific tissue composition and exceptional physical conditions of the IVD (hyperosmotic and extremely low concentrations glucose, oxygen and other nutrients) (172;173).

Several organ culture (ex vivo) models with IVDs of various animal species have been introduced to study disc mechanical function and the role of mechanical loading in DDD (174-178). These models vary in their relevance to the human situation with regard to IVD dimensions (small animals like mice and rats) (179), biomechanical properties of the animal IVD (bovine tail, lumbar porcine and sheep) (180-186), and cellular and matrix composition (rabbit lumbar disc) (172;187-189). Ideally, an ex vivo model would implement a large species lumbar IVD, comparative in biological and mechanical properties to the human IVD [41], as a precursory platform to an in vivo DDD model for follow-up studies.

Natural mechanical loading conditions of the caprine spine resemble those in the human erected spine, despite being a quadruped and therewith the predominately horizontal orientation of the spinal motion segments (190), as in vivo measurements
have shown (191). The geometry of the lumbar caprine IVD (76;192;193), biomechanical properties (39;43;44;135;194;195) and matrix content are more comparative to human IVDs (132) than discs from small animals (rats, rabbits), or tail discs from cows, pigs or sheep (196-200). Importantly, as in human IVDs, the caprine IVD lacks notochordal cells, which also makes the goat IVD comparable from an embryological and cell biology perspective (187;188;201). It is thought that due to the lack of notochordal cells, like the human IVD, the goat IVD has naturally occurring degeneration with ageing (202;203). Together with our well established goat in vivo IVD degeneration (188;204) and herniation model (205), the caprine lumbar IVD is an excellent candidate for ex vivo studies on disc degeneration and regeneration. Such a translational study platform could be utilized to answer fundamental questions in a more comparative to human model, and separate the wheat from the chaff regarding newly proposed diagnostic and therapeutic options.

**Overall aim of this thesis**

The general goal of the current thesis is to get a better understanding of how mechanical loading conditions can influence the IVDs status using an ex vivo IVD culture model. We want to know to what extent loading can be considered a stimulus and at what point it becomes detrimental to the IVD. We will examine different types of loading conditions (no load, simulated-physiological and overloading) and how they affect IVD cells, extracellular matrix and mechanical properties. We will study the IVDs response in more detail to gain knowledge on disc region specific responses and whether a certain type of mechanical loading can be linked to herniation of the IVD. Furthermore, we will utilize our ex vivo model to examine the potentials of a biomechanical evaluation method and novel MRI techniques in their ability to detect early degenerative changes in the IVD.

**Specific aims and questions**

**Chapter 2:**

*Is long-term ex-vivo culture of a large lumbar IVD feasible?*
The objective of the study in chapter 2 is to test the feasibility of ex vivo culture of caprine lumbar IVDs in the Loaded Disc Culture System (LDCS) over a 21-day period. The LDCS is a custom-build bioreactor (figure 1), designed to culture an entire large lumbar IVD. By culturing a complete IVD with cartilaginous endplates intact, we hope to conserve the IVD cells in their native environment. The LDCS allows for precise control and monitoring of oxygen- and nutrient supply, as well as (axial) mechanical loading conditions via a force-feedback loop. If culture is feasible, the LDCS will provide a platform to study the interaction between disc loading and IVD biology. Moreover, an ex vivo model may serve as a prescreening platform of future diagnostics and therapeutics prior to in vivo testing on live animal and clinical trials. We hypothesize that applying a diurnal regime of sinusoidal mechanical axial loads with adequate magnitude, along with other specific culture conditions will simulate in vivo physiological conditions and therefore maintain the goat IVD properties in culture.

Is there a need for axial loading to maintain native IVD properties in culture? What is the effect of unloading or low dynamic loading when compared to a simulated-physiological load?

We aim to characterize the IVDs response to ex vivo culture with and without loading on a biomechanical, cellular and extracellular level. We hypothesize that without an adequate axial force applied to the disc and therewith no stimulate of fluid-flow due to a lack of disc deformation, the unloaded or low dynamic loading condition will have a detrimental effect on the IVDs status.

Chapter 3:
Can mechanical overloading cause IVD degeneration?

The main function of the IVD is to transfer high magnitude axial forces, while maintaining flexibility of the spine. Loading is therefore a natural stimulus for the IVD and is even thought to be essential for maintenance of cell viability and matrix biology (206). Conversely, excessive mechanical loading evokes catabolic cellular behavior, which may trigger a cascade towards disc degeneration, i.e. loss of proteoglycans and water from the disc, with subsequent changes in mechanical
properties of the disc and further matrix breakdown (82). Whether mechanical loading is a positive stimulus or induces damage to the IVD, is dependent on the type of load applied, its magnitude, duration and frequency (120;123;207). Furthermore, it has been reported that threshold values for beneficial or detrimental effects of static and dynamic loading differ between disc regions (89;115-119). We aim to improve our understanding of the mechanobiology involved in load-induced IVD degeneration and thereby provide more integral insight in the early degenerative process. We hypothesize that both static and dynamic overloading lead to disc degeneration, resulting in changes in the biomechanical behavior of the discs’ cell survival, gene expression, and matrix structure and content.

**What is the effect of dynamic and static overloading on the nucleus and annulus region?**

We will investigate whether dynamic and static overloading have different degenerative effects on the nucleus and annulus of caprine lumbar discs. We want to know how the biomechanical response changes over time and how this is connected to cell and matrix response of the different IVD regions. We hypothesize dynamic and static overloading will have different detrimental effects on the discs’ nucleus and annulus.

**Chapter 4:**

*Is there a region (anterior, lateral and posterior) specific response to dynamic and static overloading that could explain the posterolateral predilection of lumbar hernias?*

The shape of the disc, annulus thickness and the presence of the posterior longitudinal ligament (PLL) have been described as factors contributing to the posterolateral predilection of hernias (104). However, all humans have the same lumbar disc morphology and a PLL, but not all discs herniate. Furthermore, it does not explain why many herniations in patients occur without a clear inciting moment such as heavy lifting or the combination of flexion and torsion in which a weak spot in the disc might tear, but during seemingly arbitrary loading conditions (208).
If dynamic and static overloading have different effects on the nucleus and the annulus region, there may also be a difference in effect to the anterior, lateral and posterior disc (outer annulus), which could explain the posterolateral predilection of hernia's. We will conduct a series of experiments with the LDCS to investigate the influence of strictly axial dynamic or static mechanical overloading on the various regions of the IVD. We address the question of possible location-dependent effects by analyzing the regional biomechanical response (height loss and pressure distribution) in the disc to axial loads during culture and how this influences the cells and matrix in the various disc regions over time. We hypothesize that strictly axial static overloading (as a simulation of a sedentary life-style) will affect the cells and matrix in the posterolateral region more strongly than in other regions of the intervertebral disc.

Chapter 5:

How do the poro-elastic properties of the IVD change in early intervertebral disc degeneration?

Intervertebral discs have to cope with considerable (axial) pressure even during rest. They are able to do so in a healthy state via a hyper-hydrated pressurized state of the nucleus within the constraints of a semi-porous annulus ring. The disc deformation in response to axial (un)loading resembles both a fluid-flow and solid-elastic dynamic, resulting in the unique poro-elastic behavior of the IVD as displayed during subsidence and recovery. The unique intradiscal matrix properties which enable this behavior, also rely on these same mechanical and fluid-flow dynamics to maintain their own matrix and cell properties, as it protects the disc from high peak loads or too fast deformation and facilitates nutrient supply to the cells and diffusion of waste products out of the IVD (78;83;84).

The hyper-hydrated state of the nucleus is enabled by the abundant presence of GAG molecules in the nucleus, which actively attract and hold high amounts of water in their negatively charged molecular structure. It is widely accepted that the cascade of degeneration of the IVD starts when the IVD loses GAGs from its nucleus (209). In time, this causes the disc to loose height, but must affect disc behavior prior to that, in order for height loss to occur. How the poro-elastic behavior is affected will be further elucidated in this chapter. To this end we will use our bioreactor and...
injection of Chondroitinase ABC (Cabc; GAG cleaving enzyme) to create standardized degeneration in the IVDs, as a simulation of the primary phase of disc degeneration. The LDCS will allow standardized loading and culture conditions with continuous monitoring of IVD displacement (76;210). We will assess the changes in the IVD poro-elastic behavior and use histological grading, matrix content measurements and standard MR imaging to evaluate the effects of injection on the IVDs.

*Can we use exponential fitting to identify dysfunctional disc behavior before height and water are permanently lost?*

We will characterize the deterioration of the poro-elastic properties of the IVD in response to the Cabc injection, by fitting the recovery displacement curves with a stretched exponential function. The disc behavior will be expressed by the three parameters from the fits and we will evaluate them on their capability of identifying early DDD. Outcomes will be compared to absolute displacement data (height loss) and correlated to histological grading, matrix water and GAG content measurements and Pfirrmann score. We hypothesize that the stretched exponential parameters will closely reflect the various changes in biomechanical and matrix properties.

**Chapter 6:**

*How do quantitative T2, T1rho and ADC maps change with mild IVD degeneration?*

We will use our ex vivo culture model and injection of two different concentrations of Cabc to create graded disc degeneration of the lumbar caprine IVD. High-resolution (9T) images will be obtained before and after the culture experiment. The differences between T2, T1r and ADC pre- and post-scans will determine which sequence is most sensitive to detect the various grades of IVD degeneration. We hypothesize that T1r and ADC, due to a higher spatial resolution, will have stronger signal changes when compared to T2.
Which MRI technique is superior in quantifying early (regional) IVD degeneration?

We will correlate quantitative T2- and T1rho and ADC mapping results with measured biomechanical parameters of disc behavior, histological appearance (Rutges scale for degeneration) and the absolute amount of water and PGs in the matrix after culture, to depict which quantitative MRI sequence is the best candidate to detect early degenerative matrix changes in the disc. We hypothesize that T1r will correlate strongest to actual biomechanical and matrix changes, because it is most sensitive to changes in protein-bound proton content (e.g. loss of GAGs).
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