Use of Intervertebral Disc Whole Organ Culture for Degenerative and Regenerative Studies

Graduate School for Cellular and Biomedical Sciences

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PhD Thesis

Submitted by

Svenja Illien – Jünger

from Germany

Thesis advisors

Prof. Dr. Keita Ito
Department of Biomedical Engineering,
Eindhoven University of Technology, The Netherlands

Prof. Dr. Mauro Alini
AO Research Institute, Davos, Switzerland.

PD. Dr. Stephen Ferguson
Institute of Institute for Surgical Technology and Biomechanics
Medical Faculty of the University of Bern

To my family and friends
for all their support through these years

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Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

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Summary

Use of Intervertebral Disc Whole Organ Culture for Degenerative and Regenerative Studies

Low back pain (LBP) is the primary cause of disability in the active age group in Western society, with a lifetime prevalence estimated at 80%. In many cases, low back pain is related to intervertebral disc (IVD) degeneration. Although its etiology is still unclear, a potential cause for degenerative changes is diminished nutrition. The IVD is the largest avascular organ in the human body and the main nutrient supply is provided by the capillary buds ending in the adjacent osseous vertebral endplates (EPs). Hence, the cells in the disc must rely on diffusive and convective transport of nutrients and metabolites across a rather large distance; thus, the nutrient transport situation for the disc cells is precarious already at birth. During aging, the cartilaginous endplates of the disc calcify and become less permeable to nutrients, which is believed to result in even lower density of viable cells. Beside nutritional shortage, there are also indications that dynamic loading can be deleterious to the disc. Dynamic compressive loading has been shown to lead to degenerative changes to the disc. In several epidemiological studies, the exposure to whole body vibration has also been related to LBP. Particularly vibration in the 4 to 10 Hz range has been identified to stimulate degenerative processes. In this thesis, the aim was to develop an *in vitro* system for culturing whole intervertebral disc (IVD) explants with "simulated

physiological" loading through intact endplates (EPs). Based on this model, the response of disc cells to nutritional challenge by limiting nutrition and further determined the effects of high (10 Hz) frequency loading was investigated. In a next step, the aim was to investigate, by means of this novel organ culture model, whether metabolically and mechanically challenged discs release factors that may attract human BMSCs. The central hypothesis was that limiting nutrition as well as high frequency loading have detrimental impacts on IVDs in terms that they might impair the disc cells in an additive or synergistic manner. It is known that injured tissues release factors that stimulate progenitor cells to migrate to the injured site. Therefore, a further aim was to investigate if this also applies to IVDs.

In the first part of the present work, the whole organ culture model was established. Cell viability as well as matrix proteins at the gene expression level was maintained up to 21 days in culture when cultured under sufficient nutrition. In contrast, limiting nutrition caused increased cell death (~55%) within the first days and thereafter remained stable during 21 days. Despite the substantial reduction in living cells, the nutritional challenge was not enough to provoke other indications for disc degeneration to occur at the gene expression and protein level during a short and mid term whole organ culture. From this it was concluded, that under physiological conditions, the metabolic activity of cultured disc could be maintained for up to three weeks. Whereas, under declined nutritional supply, first signs of early degenerative processes could be induced. However, limiting nutrition for 21 days was not enough to induce extra cellular matrix degeneration of the disc. It was supposed that due to the relatively low cell density within the IVD, sufficient cell-to-cell signaling might have been limited. If the direct environment of the cells would change, e.g. due to physical stress, one could expect an earlier response of the cells.

Therefore, in the second part of the thesis, the effect of high frequency load combined with limiting nutrition was investigated. In this work, whole IVDs were exposed to high frequency (10Hz) loading in order to mimic deleterious

vibration, which then was compared to "physiological" (0.2Hz) loading. Additionally, the influence of high frequency load on IVDs cultured under "limited" nutrition conditions was also investigated to reproduce the experience of potentially detrimental loading in discs suffering from a compromised nutrition state, e.g. due to EP calcification. Culture under either high frequency loading or limited glucose conditions led to a significant drop in cell viability. Combining both treatments resulted in an additive increase in cell death of the disc cells and in an increase in MMP13 gene expression. These findings, extrapolated to the human situation, could indicate that when subjects with IVD nutrition problems are additionally exposed to high frequency vibration sources (~10 Hz) they might be more likely to suffer from disc degeneration with respect to increased cell death. However, neither frequency nor limited glucose affected cell metabolism, regarding degradation of the extracellular matrix. From this it was presumed, that a longer culture period, e.g. several weeks under limited nutrition combined with high frequency might be required to create degeneration of the extra cellular matrix within the disc. The presented culture system provides nonetheless the opportunity to study the detrimental implication of frequency load combined with reduced nutritional supply compared to "physiological" cultured discs, which apparently induced early degenerating discs.

Based on this outcome, in the last part of this thesis the aim was to investigate in whole organ culture whether metabolically and mechanically challenged discs release factors that may attract human BMSCs; since it is known that cells from injured tissues release cytokines and mediators that are involved in the healing process. Compared to IVDs cultured under "simulated-physiological" conditions, the number of BMSCs that migrated towards the center of the disc was increased in "degenerating" IVDs. Additionally, the media of "degenerating" discs demonstrated chemo-attractive activity towards hBMSCs. The results of this work show that "degenerative" conditions may induce the release of trophic factors that promote the recruitment of BMSCs. Since regenerative effects of

attracted BMSCs can be envisaged, this finding has implications for therapeutic delivery of BMSCs and endogenous progenitor cell activation.

In summary, the IVD culture system presented in this thesis provides the unique possibility to investigate degenerative and regenerative mechanisms in a mid-term whole organ culture. It provides a suitable base for further tissue engineering investigations e.g. for testing cell therapy and scaffolds in a native ECM without going into the difficulties of in vivo experiments.

Chapter 1

General Introduction

1.1 Motivation

Low back pain (LBP) is the primary cause of disability in the active age group in Western society. With a lifetime prevalence estimated at 80%, it has an enormous socio-economic impact.[1,2] Although intervertebral disc (IVD) degeneration is not directly associated with LBP in all cases,[3] there is mounting evidence of a link between the degenerate IVD and clinical symptoms.[4] IVD degeneration has been related to altered spine biomechanics due to decreased nucleus pressure, reduced tensile stiffness, increased compressive and shear modulus, and increased deformability of the disc.[5-8] It has also been associated with sciatica and disc herniation.[9] However, the underlying causes for disc degeneration (DD) are multifactorial. Genetic factors[10] and aging[11] are known predisposing factors for DD and also mechanical loading has been shown to play a role.[12-14]

The IVD is the largest avascular organ of the human body. Therefore, the cells must rely on diffusive and convective transport of nutrients and metabolites across a rather large distance.[15,16] Conditions that further limit the nutrient supply into the disc, e.g. smoking, vascular disease, and vascular deficiency[11,17], have been associated with higher incidence of DD and calcifications in the cartilaginous endplates (EPs)[18], occluding the osseous EP openings, have been correlated with the severity of DD.[19]

There are also indications that dynamic loading can be deleterious to the disc. Dynamic compressive loading has been shown to lead to degenerative changes to the disc, such as breakdown of the extra cellular matrix (ECM).[12,13] In epidemiological studies, exposure to whole body vibration (WBV) has also been related to low back pain (LBP). Particularly, vibration in the 4 to 10 Hz range was found to stimulate degenerative processes.[20,21] It is suggested, that subjects experiencing potentially detrimental loading and suffering already from a compromised nutrition state, e.g. due to EP calcification, might be even more prone for developing disc degeneration.

To study degeneration and evaluate regeneration strategies, various models have been developed in many species such as mice, rats, sand rats, rabbits, dogs, sheep, pigs, goats and apes. Even though there is a common developmental pathway for the IVD in all mammals, there are small but crucial differences between species, e.g. in the composition of cells that populate the nucleus pulposus (NP). In humans, the notochordal cells, remnants of the embryogenic notochord, start to disappear already shortly after birth, whereas pigs, rats and mice retain them throughout their life. The NP of rabbits contains large numbers of notochordal cells at least until 12 months of age.[22] One reason for retention of those cells might be the relatively small disc size, eliminating concerns regarding nutrition shortage. On the contrary, the disc dimensions of cows and sheep are similar to the human IVD. In addition, both lose their notochordal cells shortly after birth – therefore they might be more reliable models for disc research.

To study the influence of nutrition on DD *in vivo*, Krebs *et al.*[23] performed vertebroplasties in adjacent vertebrae in the ovine lumbar spine. The changes in the disc were modest or absent and neither perfusion disturbance nor solute concentrations were documented, indicating the inherent difficulty to control solute transport *in vivo*. In *in vivo* experiments, the whole body is responding to the specific treatment, implying both advantages and disadvantages. The biochemical and biomechanical factors, stimulated by the intervention, are difficult to control. It is difficult to exclude that no other pathway covers the impact of a specific treatment or that a second trigger causes the observed response to a defined stimulus.

In consequence, for some questions, *in vitro* culturing systems are more appealing, since better control of single factors is possible and they are generally less expensive. Many *in vitro* studies have focused on the behavior of disc cells in artificial three-dimensional environments.[24-26] In all of these investigations, cells were removed from their native complex three-dimensional environment and underwent cell passaging. Even though a specific parameter can be investigated in

detail, one should keep in mind that removal from their native, highly specialized, extracellular environment doubtlessly affects the cell behavior.[27,28]

An important link between *in vitro* and *in vivo* studies are explant models, since controlled *in vitro* conditions can be applied to cells that are kept in their native surrounding. In recent years, various explant models have been developed with different approaches and species.[29-32] As the main challenge of IVD explant culture is the disc swelling when placed in standard culture medium[32], several procedures have been applied to prevent tissue swelling. Kim *et al.* cultured rabbit lumbar discs with endplates (EPs) under dynamic compression, whereby DNA as well as proteoglycan content was maintained for up to 2 weeks.[33]

Haschtman *et al.* cultured rabbit IVDs with EPs in DMEM/F12 standard medium under free swelling conditions. The authors report that cell viability could be maintained for at least four weeks without losing structural integrity and matrix composition. Nonetheless, they observed a degenerative gene expression pattern and concluded that their model could be used to study DD.[35] In a subsequent study, rabbit IVDs were cultured with EPs under diurnal hyperosmotic loading. However, they found that diurnal hyper-osmotic stimulation of a whole-organ culture partially diminished the matrix gene expression profile as encountered in degenerative disc disease.[34]

All these studies used discs from rabbit that are much smaller than human lumbar discs; therefore, as discussed earlier, any diffusion limitations might have been minimized in these systems. Moreover, the maintained matrix composition and structural integrity may depend on the presence of notochordal cells, which have a more active metabolism than NP cells.[36,37]

Ohshima *et al.* cultured bovine IVDs under 5-kg static load in order to maintain *in vivo* hydration levels. They measured changes in metabolic activity in response to varying load magnitude. Even though that study was limited to 12 hours and used discs devoid of endplates, the system appeared to be promising, especially in terms of its applicability for mechanobiology studies of the

intervertebral disc.[38] Lee *et al.* evaluated a similar system for longer-term culture, in which they applied static load to the disc. [39] Although they could maintain the cell viability up to seven days, glycosaminoglycan (GAG) synthesis rate dropped by 70% to 80% after only two days of culture. They suspected that this drop might have been a result of static loading, since in vivo experiments have indicated stimulation of anabolic proteins with higher frequency loading. Another purpose of their study was to explore the possibility to culture discs with EPs, which would maintain the biomechanical integrity of the disc by anchoring the annulus fibers and preventing dislocation of the nucleus pulposus. However, they found that culturing IVDs with endplates drastically reduced cell viability, especially in the nucleus. This was likely due to blood clotting, since the transport routes through the EP were not re-opened.

Based on these findings, Gantenbein *et al.* cultured ovine IVDs with EPs that had been treated with systemic anticoagulation and evacuation of the endplate vasculature, i.e. by using weak acidic treatment or jet spaying cleaning techniques.[40] Additionally, they applied cyclic diurnal loading and could maintain cell viability and GAG synthesis rate for seven days. On the other hand, gene expression data indicated possible catabolic changes in cell activity. However, cyclic diurnal loading cannot sufficiently simulate the in vivo situation. Therefore, a novel bioreactor was developed to be able to apply a physiological loading magnitude and frequency superimposed on the diurnal cyclic loading. With this system, our motivation was to simulate physiological loading and extend the culture time from seven days up to three weeks. We also aimed to investigate the effect of limited nutrition and frequency load to establish a model for degenerating discs. The set up of such a model also allowed us further investigations into regenerative medicine. Therefore, the aim of the last part of this project was to explore the possibilty that stressed disc cells could attract bone marrow derived mesenchymal stem cells (BMSCs) to migrate (homing) at the damaged site of IVD.

1.2 Objective and Hypothesis

Of the various factors related to DD, we were, in a first step, most interested in limited nutrition because it might initiate a cascade of events giving rise to the onset of DD. Secondly, of the mechanical causes for disc DD, such as load magnitude, duration and frequency, we were particularly interested in frequency because it has been implicated in many occupational activities such as vibration during truck or heavy-equipment driving.[41,42] It is assumed that high frequency load leads to decreased nutritional diffusion through the disc; due to the time span between loading periods which might be too short to allow significant fluid flow. Hence, we hypothesized that a) high frequency loading will alter disc degeneration and b) that the combined effects of reduced nutrition and high frequency loading would affect the IVD cells in an additive manner with limited nutrition leading to more severe DD. Finally in the last part, we focused on the possibility to use our organ culture system to evaluate potential regenerative mechanisms. It is know that following an injury, the body will react to the insult by releasing cytokines and mediators, which are involved in the healing process. One important function of these factors is the recruitment of progenitor cells, which then contribute to tissue repair. Therefore, in a third step we used our *in vitro* IVD explant model to test the hypothesis that BMSCs are recruited by nutritionally or mechanically challenged discs providing endogenous regenerative capacity to treat early DD.

The following hypotheses were tested:

1. DD can be initiated by limited nutrient during a short (seven days) and mid-term (three weeks) culture.
2. Higher-frequency (as compared to simple diurnal cycles) mechanical stimuli can alter the rate of DD. Furthermore, the combination of high-frequency compression and decreased nutrient supply can have an additive effect leading to progressive DD.
3. Metabolically and mechanically challenged discs release trophic factors that may attract human BMSCs.

1.3 Outline of the thesis

The overall goal of this thesis is to develop an intervertebral disc whole organ culture system for degenerative and regenerative investigations, focusing on the impact of nutritional shortage and high frequency load. The main body of this thesis has been written in the form of individual manuscripts. Each article addresses one of the three hypotheses stated above. The third manuscript is currently in preparation and additional experiments are planned.

Chapter two provides more detailed background information on the human spine, including the composition of the intervertebral disc and its tissue properties, the development of degenerative disc disease, potential reasons for the same and current as well as prospective treatments for low back pain, completed by a short overview about disc degeneration models. In chapter three, the general methodology is described, summarizing the establishment of the organ culture bioreactor and the harvesting of intervertebral discs. Chapter four and five concern the maintenance of intervertebral discs in whole organ culture under physiological conditions and under detrimental impacts of diminished nutrients and deleterious loading. In chapter six the degeneration model is used to investigate the homing potential of BMSCs in stressed intervertebral discs. Chapter seven provides the general discussion on the relevance of whole organ cultures and the clinical significance of the present work; it further goes into the limitations of the studies and presents a general conclusion as well as perspectives for future investigations.

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Chapter 2

Background

2.1 The human spine

The human spine consists of 34 vertebrae that are divided in 5 sections: 7 cervical vertebrae, 12 thoracic vertebrae, 5 lumbar vertebrae, 5 sacral vertebrae and 5 coccygeal vertebrae. Viewing the spine from the side it appears as a "double-S". The cervical spine is bent convex anteriorly (lordosis) whereas the thoracic part is bent convex posteriorly (kyphosis). The lumbar section is again lordotic followed by the sacrum and coccyx that are kyphotic. This special form of the human spine provides compliance and helps to protect the body, by minimizing shock loading e.g. while walking or when exposed to vibrations. From the 34 vertebrae that build the spine, the five vertebrae of the sacrum and the coccyx are fused. Therefore, the spine is often defined as being composed of 24 free vertebrae; separated by 24 intervertebral discs (IVD) that lay ventral to the spinal canal. Besides its very important weight-bearing function, the spine is essential for permitting movements in all directions. Hence, the spine is unique in its structure in order to accomplish its functional demands that are met by muscles, ligaments and intervertebral discs.

2.2 The healthy IVD

The IVD consists of three major substructures: the sturdy, collagenous annulus fibrosus (AF) surrounds the highly hydrated, gelatinous nucleus pulposus (NP). Both lay between two cartilaginous endplates (CEPs; Figure 1) The composition of each structure is distinct and suggests that each component has a specific mechanical role. The discs in the lumbar region of the spine are kidney shaped in transverse cross section, with the nucleus centered posteriorly (Figure 2).

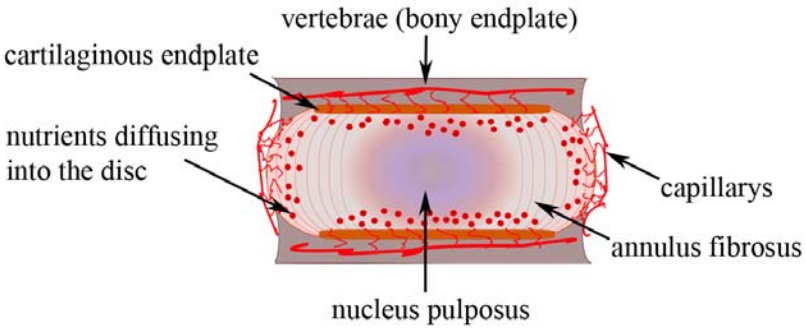

Figure 1: Sagittal section of the intervertebral disc; the central NP is surrounded by the lamellae of the AF; both are covered cranially and caudally by cartilaginous EPs that are attached to the vertebral bodies

The AF is a highly oriented fibrous tissue with a lamellar structure of collagenous fibers, which form concentric, parallel rings. The collagen fibers lay 60° to the vertical and are arranged in opposite directions in successive layers of 10-25 lamellae[1] (Figure 2). The outermost lamella of the AF is linked to the posterior longitudinal ligament and inserts into the vertebral body via Sharpey's fibers. The innermost AF merges with the NP and the collagen fibers continue vertically through the EP. [1]

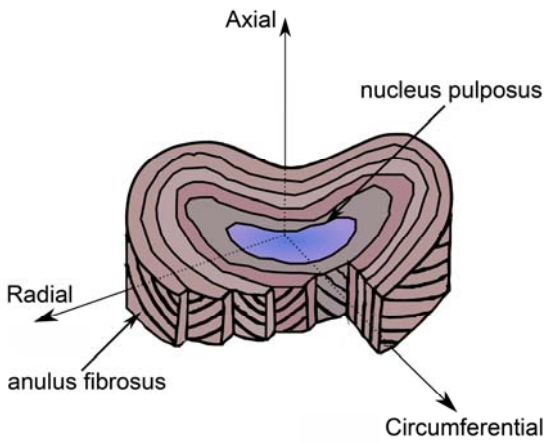

Figure 2: Schematic of a lumbar intervertebral disc with cylindrical polar coordinate system

The NP is more gelatinous, highly hydrated and composed mainly of proteoglycans (PGs). It is more randomly arranged, less tough and mechanically stable than the AF. The boundary between both tissue types is a smooth transition and known as the transition zone. The CEPs that cover this structure are composed of hyaline cartilage. In early life, the CEP occupies more than 50% of the IVD and has many large vascular channels, which clear away by the age of 4-6 years.[2] With a water content of 70-80% and PG content of approximately 7% of the dry weight, the composition of the CEP is similar to that of articular cartilage.[3] The region above the outer annulus as well as adjunct to the vertebral body contains more collagen but less PG and water than the CEP close to the disc, above the NP.[3] As the disc is the largest avascular structure in the human body, [4] nutrients and metabolites have to diffuse through the cartilage EP or through the outer AF to reach the cells of the disc center[5,6] (Figure 1) . As a result, oxygen tension and glucose concentrations within the central region of the disc are low and lactic acid concentrations are high.[7] Because of a low cell density (4'000-9'000 cells/mm^3)[8] and low cell activity, the IVD has inherently low self-repair capabilities. During a lifetime, the CEP reduces to a thin avascular layer with eventual calcification in adolescence;[9] thereby, it becomes less permeable, correlating with an increased risk of disc degeneration due to the even further decreased nutrition supply.[10]

2.3 The extracellular matrix

As previously mentioned, the extracellular matrix (ECM) of the IVD consists mainly of PGs and collagens with distinct differences in composition of both tissue types. Progressing from the AF to the NP, the PG content increases accompanied with a concomitant decrease for collagen. With 80% of the dry weight, collagen forms the integral part of the ECM in the AF; in the NP, it accounts for 20%.[11] Collagen I and II make up 80% of the total collagen, with the ratio of collagen I and II being inverted: The AF contains mainly type I and small amounts of type V, VI, IX and XI; whereas the NP mainly consists of type II

collagen (approximately 80%) and lower quantities of VI, IX and XI. Collagen type III is located only in the NP and the inner AF.[12] It is assumed that the minor collagens might influence the interactions between collagen fibrils and PGs and thereby regulate collagen I and II fibrillogenesis in the IVD.[13,14] In the NP, PGs are the predominant molecules with 70% of the dry weight in juvenile discs[15] and 27-times more abundance than collagens; whereas, in the AF PGs are nearly absent.[16-19] PGs have the mechanical property to withstand compressive stresses due to daily loading. The largest and most important PG is aggrecan. The large proteoglycan forms large aggregates by binding to hyaluronan (Figure 3).

Aggrecan consists of a central core to which 30 keratan sulphate (KS) and more than 100 chondroitin sulphate (CS) chains are attached, flanked by two globular domains in the N-terminus (G1 and G2) and one in the C-terminus (G3). G1 binds non-covalently to hyaluronan, stabilized through a link-protein, a small globular glyco-protein. The fixed chondroitin and keratan sulfate (SO_4^{2-}) and carboxyl ($COOH^-$) groups ionize at physiological conditions becoming negatively charged,[20] which is important for imbibing water. Besides aggrecan, also smaller PGs (biglycan, decorin, fibromodulin and lumican) are present in the IVD; they are thought to influence the cellular deposition and collagen assembly.[21,22] Another component found in the IVD is elastin. Its elastic fibers are randomly located in the whole disc and thought to restore the tissue shape after deformation. In the AF, it is located in the interlaminar spaces, laying parallel to collagen fibers and forming cross-bridges between the layers of the outer AF.[23]

Besides altered collagen IX genes, also variances in the expression of aggrecan have been found to be associated with an increased risk for disc degeneration.[62] The CS domain of the large proteoglycan consists of two regions, CS1 and CS2 (Figure 3). A polymorphism within the allele for CS2 leads to aggrecan core proteins that vary in length, affecting the number of CS chains that can bind. Shorter aggrecan molecules might have lower capacity for hydrating the disc, which in turn affects its mechanical properties. In 1999 Kawaguchi *et al*.[62]

described that an over presentation of alleles coding for short CS domains was found in individuals with multilevel disc degeneration.

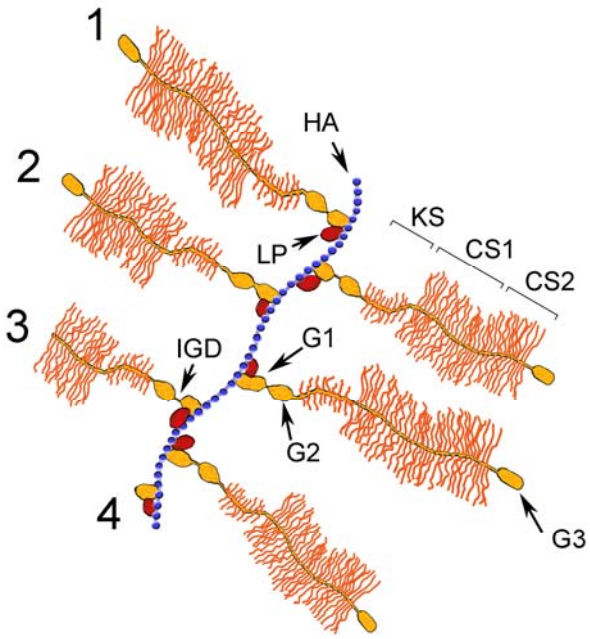

Figure 3: structure of aggrecan: CS = chondroitin sulphate; G1-G3 = globular domains; HA = hyaluronan; IGD = inter globular domain; KS = keratan sulphate; LP = link protein. Aggrecan contains three globular domains (G1–G3) that are separated by an IGD, KS and CS. The CS1 domain possesses a variable number of tandem repeats, so that individuals may have aggrecan core proteins that can be long (1) or short (2). Disc degeneration involves proteolytic cleavage of the aggrecan core protein, often within the CS2 domain or IGD, resulting in fragments enriched in the CS1 domain (3) or the G1 domain (4).

2.4 Tissue properties

The NP allows the inflow of water and ions but prevents the motion of fixed charges of the PGs. The disc consists of 65-90% water and, even though this extrafibrillar fluid component is mobile, its motion is limited due to the low permeability of the disc tissue, creating a frictional drag between the solid and the water phases.[24] Due to this, a high osmotic potential arises and large amounts of water are drawn into the NP, leading to expansion of the ECM. In normal human adults, the osmolarity of the disc ranges from c. 400 mOsm, when the water

content is highest to c. 550 mOsm after maximum daily fluid expression.[25] Water, nutrients and metabolites may be transported into and out of the disc through the EP and the outer AF. The EP provides a barrier for the water outflow of the disc, resulting in a non-linear flow-direction-dependent resistance to fluid flow.[26,27] The disc reversibly deforms under stress and the water content is dependent on the balance between the swelling pressure of the NP, the collagen fiber tension in the AF and the applied load on the spine. During one day of activity and rest, the total amount of fluid exchanged varies from approximately 10-20% of the total disc volume,[28,29] with an estimated height loss of 7 mm (0.39% of the body height) after the active phase.[30]

2.5 The IVD cells

The NP forms from the embryonic notochord and is initially populated by notochordal cells; while the AF and CEP are formed by the mesenchyme.[31] In humans, after birth the number of notochordal cells decreases rapidly from approximately 200/mm^3 at six weeks of age to 100/mm^3 at one year. At the age of approximately four years, no notochordal cells remain in the NP.[32] In adulthood, the human NP is populated by cells that morphologically and phenotypically resemble chondrocytes.[33] They are rounded and enclosed in a capsule.[14] However, compared to chondrocytes, NP cells contain cytoplasm-filled processes.[34]

AF cells are phenotypically distinct from NP cells. In the AF, cells are more fibroblast like, being thin and elongated, and extend along the collagen fibers of the lamellae. Besides their morphology, they also differ in their metabolic activity, e.g. NPC synthesize glycosaminoglycans (GAG) more rapidly than AFC with a ratio of 3:1 for ^{35}S incorporation into PG.[35-38] Type II collagen is also more highly produced in the NP than in the AF.[39]

2.6 Degenerative disc disease (DDD)

Low back pain (LBP) is one of the most common health problems for young to middle aged individuals in modern western society,[40] and as such, it has a tremendous socio-economic impact.[41,42] Although degeneration is not symptomatic for LBP in all cases,[43] there is mounting evidence of a link between the degenerate IVD and clinical symptoms. For proper function, the disc depends upon maintenance of a delicate balance of molecular composition and structural integrity (see 2.4). A disturbance of that balance might result in breakdown of the ECM. Radiographically, degenerated discs show altered appearance going along with a collapse of the disc space, bulging, and EP sclerosis, sciatica or IVD herniation[44]. These herniations can subsequently compromise the neural canal and impinge upon or directly irritate the nerve roots and posterior longitudinal ligament (Figure 4). It has been shown that IVD degeneration affects spine biomechanics due to decreased nucleus pressure, reduced tensile stiffness, increased compressive and shear modulus, and increased deformability of the disc.[45-47] This may cause severe chronic lower back pain and/or sciatica, which is one of the most economically significant orthopedic disorders in industrialized society.

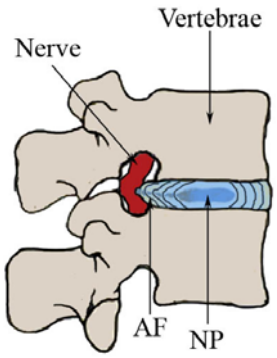

Figure 4: Herniation of the nucleus pulposus can compress or irritate the nerve roots. The resulting pain and numbness can be obstructing, yet few therapeutic options exist

With increasing degeneration, the entire disc undergoes significant changes in composition and color, going along with increased fiber content in the NP and altered fibers in the AF (Figure 5).[9,17,48]The structure of the proteoglycans changes (Figure 3) and the amount of PGs decreases from approximately 60-70% in the healthy disc to less than 20% in severe degenerated disc, going along with decreased fixed charge density in the tissue resulting in lower osmotic pressure.[49]

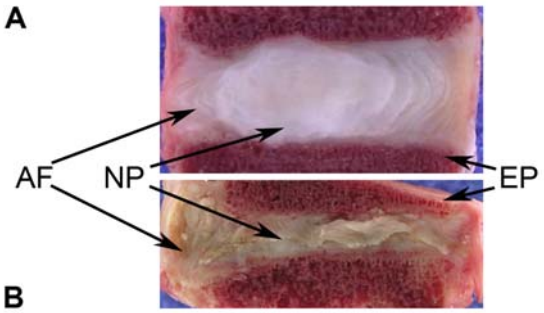

Figure 5: Sagittal section of the intervertebral disc; **A** = healthy IVD, **B** = degenerated IVD; AF = annulus fibrosus, NP = nucleus pulposus, EP = end plate. Note that in the degenerated disc both tissues are altered and not distinguishable

The disc becomes less hydrated and is unable to maintain its water content under load. This, in turn, leads to changes in the mechanical, chemical and electrical properties of the disc.[15,45,49] The distribution of collagens is also affected in degenerated discs: in the inner annulus the relative amount of collagen II decreases.[15,50] Other signs for degeneration are slight decreases of newly synthesized aggrecan and collagen II, loss of lamellae in the annulus and an increase of the remaining layers accompanied with inter-bundle spacing.[1,15] The EP is affected as well, as its thickness becomes irregular with local defects which might result in calcification.[9,51] The stress distribution in the AF and EP will change when the altered IVD cannot fully withstand the load anymore, resulting in mechanical damage[52] and likely in compensatory contraction of the surrounding muscles. The final reasons for LBP are miscellaneous: it might be the IVD itself, the surrounding muscles that spasm, nerves and blood vessels that are

incorporated into the outer AF. Therefore, it is important to fully understand the nature of LBP, the mechanisms of disc degeneration and its triggers.

2.7 Potential reasons for disc degeneration

Degeneration of the IVD is more common and occurs earlier than degeneration of other cartilaginous tissues. As such, ageing is without doubt one of the most important factors linked to disc degeneration.[9,53,54] In addition to age, genetics has been suggested to predispose to accelerated degeneration leading to disorders of the IVD.[55] In twin studies it was found that DDD often is based on a genetic component, with a heritability of over 60%.[56-58] Mutations in the genes for collagen IX, aggrecan, cartilage intermediate layer protein (CILP) and Asporin have been found to be related to disc degeneration. Although collagen IX is only a minor component in the ECM of the disc, a mutation in this gene can have severe impact on the disc structure. The fibrillar collagens I, II and IX are similar in size and structure, since all contain a large triple-helical domain with about 1000 amino acids per α chain. However, the minor collagen IX is somewhat different from the major collagens, as it contains four short non-triple-helical domains (NC1-NC4). Within the NC3 domain of the α2 chain collagen IX has an attachment site for a glycosaminoglycan (GAG) side chain that can be either chondroitin or dermatan sulphate. Therefore, collagen IX can be classified as a proteoglycan.[59] The molecule is located along the surface of collagen II, interacting through covalent lysine cross-links Figure 6).[60] As a result, collagen IX might be located at the surface of the fibrils with short COL3 and globular NC4 domains sticking out. Thus, it could act as a macromolecular bridge between collagen fibrils, and between collagen fibrils and other ECM molecules. Hence, mutations in collagen IX binding sides could affect the long-term stability of the ECM.[61]

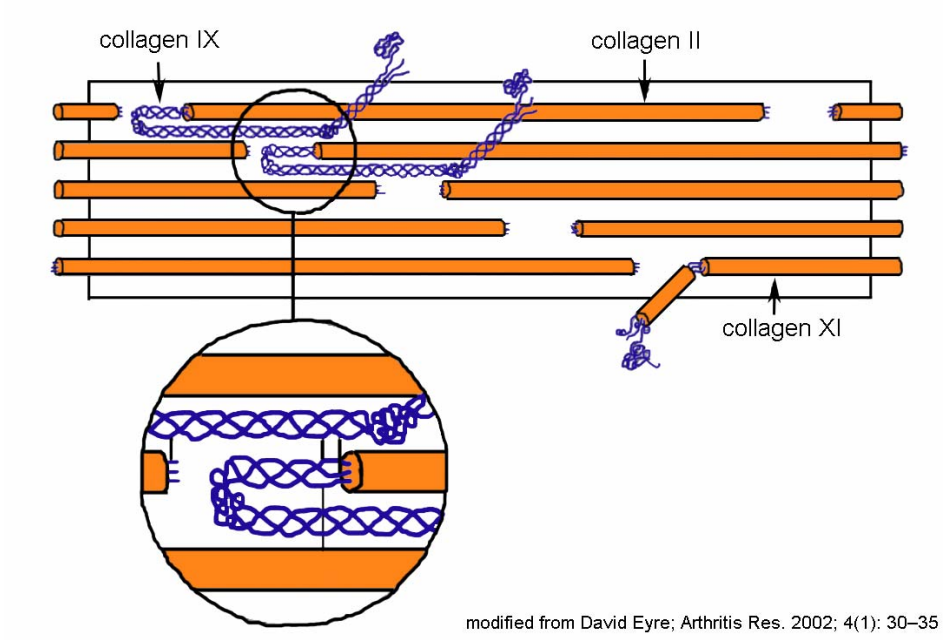

Figure 6: Molecular model of the collagen type IX fold and interaction site with a collagen II microfibril that can account for all known cross-linking sites between collagen II and IX molecules.

Another cause for DD can be a single nucleotide polymorphism (SNP) in CILP, a gene encoding the cartilage intermediate layer protein. The SNP results in expression of a Cytosin instead of a Thymin, leading to increased binding capability to TGF-β, which is important in maintaining ECM proteins in the IVD.[63,64] Seki *et al*. found that in absence of CILP, TGF-β1 induces the expression of collagen type II and aggrecan. Whereas, in presence of CILP the induction is inhibited.[65] One possibility to control TGF-β activation is through the ECM protein decorin. Decorin binds TGF-β and directs its accessibility to receptors.[66,67] Supersession of decorin by CILP may interfere physically with the binding of TGF-β to its receptor by sequestering it. The aberrant increased inhibitory effects of the mutated CILP might perturb the balance of TGF-β over IVD tissue maintenance.

Asporin (ASPN), another ECM protein, is also known to bind and negatively regulate TGF-β activity.[68] ASPN belongs to the small leucine-rich proteoglycan family (SLRP). Its name reflects the aspartate-rich amino terminus and the overall similarity to decorin. However, asporin is not a proteoglycan. Instead, it contains a unique stretch of aspartic (D) acid residues in its amino-terminal region, including a polymorphic region with a number of consecutive D residues varying from 11 to 15. The effect on TGF-β activity is allele-specific, with the D14 allele resulting in greater inhibition than other alleles and being over-represented relative to the common D13 allele in individuals with osteoarthritis (OA) or DD.[68-70] Taken together, mutations in different genes might lead to altered matrix composition directly or affect the TGF-β activity which than results in alteration of the ECM.

Finally, because of their genetic background, some individuals may be predisposed to developing DD.[55] A fall in nutrient supply, going along with impaired diffusion of glucose, oxygen and waste products, could reinforce disc degeneration by creating an acidic and low-oxygenated environment that would be deleterious for maintaining cell viability and macromolecular synthesis,[71,72] especially in the prestressed disc. The nutrient supply to the NP can be disturbed at several points. Disorders affecting the blood supply can lead to degenerating discs and back pain. Thrombophilic and hypofibrinolytic disorders as sickle cell anemia,[73] Caisson disease and Gaucher disease[74] might block the capillaries and lead to adverse environment for the disc. Moreover, besides shortage of the blood supply, also sclerosis, going along with calcification of the EP, might inhibit nutrient transport into the disc center.[51] Another trigger for DD might be abnormal loading, which could amplify the risk for degeneration of vulnerable discs. It may result in fatigue failure of the ECM, leaving the cells more susceptible to mechanical loads.[75] The relation of whole body vibration (WBV) to low back pain (LBP) has been widely shown in epidemiological studies. Particularly vibration in the 4 to 10 Hz range was found to be detrimental for the IVD.[76,77] This may be due to resonance. The natural vibration frequency of the human body ranges from

3 to 14 Hz,[78] with the higher frequencies representing bending vibration of the upper torso with respect to the lumbar spine.[77] Therefore, the natural frequency of the human body and that of heavy machinery (e.g. tractors, cranes, mowers, graders) and hand machines (e.g. jackhammers, impact wrenches) are similar.[78,79]

The above-mentioned triggers finally result in a disturbance of the equilibrium between matrix synthesis and degradation.[80] One family of degrading enzymes in the IVD are matrix metalloproteinases (MMPs). MMPs are synthesized as inactive zymogens and become activated by enzymatic cleavage. Their activation is furthermore regulated by endogenous inhibitors, the tissue inhibitor of metallo proteinases (TIMPs). MMPs can be divided in four groups of enzymes: collagenases (e.g. MMP1, -7, -8, -13) gelatinases (e.g. MMP2, -9), stromelysins (e.g. MMP3) and membrane type (MT) MMPs.[80] Additionally, also aggrecanases (ADAMTS), especially ADAMTS 4+5[81], have been identified to be involved in the degradation of aggrecan in the IVD. Collagenases are able to cleave the triple helical part of collagens I, II and III. In particular, MMP13 has been described to cleave collagen type II very efficiently. Moreover, it also cleaves gelatin with greater efficiency than the other MMPs and therefore degrades the initial cleavage products even further.[80] Thus, MMP13 might be important in IVD degeneration characterized by collagen II loss in the ECM.[81] Besides MMP13, also MMP 7 (Matrilysin, PUMP 1) expression has been reported in herniated IVDs.[82] As MMP13, MMP7 efficiently cleaves collagen type II.[83] Moreover, it also is known to cleave aggrecan[84] and is capable to activate a number of MMPs and catabolic cytokines.[81,85-87] Because of its truncated form[87] MMP7 also is more resistant to inhibition by TIMP1 than the other MMPs. TIMP1 is known to increase with the increased production of a number of other MMPs.[81] Therefore, the resistance of MMP7 to TIMP1 could result in a more active role for MMP7 in IVD degeneration.[88]

Two of the activated cytokines are the families of interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). It has been shown, that the IL-1 family is key to initiating production of the matrix degrading enzyme cascades that lead to matrix

loss and reduction in disc height.[89] TNFα is known to have proinflammatory properties, possibly modulating cellular phenotypes. The expression of TNFα in adult discs has been found to be associated with DD.[86] Its occurrence in adults of more advanced age suggests that TNFα is not involved in the initiation of disc degeneration, but may be associated with further promotion of degenerative disarrangement and pain induction.[86]

2.8 Current treatments of LBP

Today, treatments are more for pain suppression than for treating the underlying pathology. Conservative treatment for back pain may include rest, use of anti-inflammatory medications, and physical therapy.[90] After a patient has undergone conservative treatment for at least six months with poor results, surgical intervention might become necessary. Surgical modalities include interbody fusion, total disc replacement (TDR) or NP replacement (NPR).[91] Interbody fusion is one of the most commonly performed procedures for degenerative disorders of the lumbar spine. Two or more vertebrae are fused when gross instability of the spine segment is detected. After discectomy, supplementary bone tissue (either autograft or allograft) is used in conjunction with the body's natural osteogenic processes, stabilized by screws, rods or plates. This procedure is used primarily to eliminate the pain caused by abnormal motion of the vertebrae by immobilizing the vertebrae themselves. Some newer technologies are being introduced which avoid fusion and preserve spinal motion. TDR and NPR are being offered as alternatives to fusion, but have not yet been adopted on a widespread basis. Their advantage over fusion has not been well established. For clinical application, effective treatments to restore the degenerated intervertebral disc are not yet available.[92,93]

2.9 Prospective regeneration strategies

In recent experimental studies, various biological strategies to arrest disc degeneration have been explored. They include the application of growth factors and cytokines, gene therapy, scaffold and cell transplantation of disc or mesenchymal stem cells. [16,94-97]

The principal function of growth factors (GFs) is to increase the cellular activity of degenerating IVDs. GFs are bioactive molecules that are important for the formation and maintenance of tissues.[97] Mainly members of the transforming growth factor beta (TGF-β) superfamily, like TGF-β1[94] and osteogenic protein (OP1)[98] are investigated. In New Zealand white rabbits, Masuda *et al.*[98] investigated the in vivo ability of OP-1 to restore disc degeneration, which was created by anular puncture with a needle. The injection of OP-1 induced a restoration of disc height at 6 weeks, which was sustained up to 24 weeks after the injection. However, one should not disregard, that treatment with osteogenic growth factors could eventually result in heterotropic ossification. Wysocki et al. recently reported ectopic ossification of the triceps muscle after application of OP-1.[99]

Beside this, due to the still relatively short half-life of GFs, the use of gene therapy seems to be a promising alternative. By genetically modifying IVD cells to induce the GF endogenously, the effect of the factor can be extended.[100] Already in 1998, Nishida *et al.*[100] showed, that NP cells could be efficiently transduced *in vitro* by an adenoviral vector carrying the lacZ gene. *In vivo*, injection of Ad-lacZ into the NP resulted in the transduction of a considerable number of cells that persisted for at least 12 weeks. They suggested that the adenoviral vector might be suitable for delivery of appropriate genes to the disc for the treatment of spinal disorders.

The underlying idea for using scaffolds is to retain cells in a specific area and emulate the ECM of the disc; resulting in an environment that is capable for cell adhesion, proliferation and synthesis of functional tissue.[101] Recently

O'Halloran *et al.*[102] developed an enzymatically cross-linked, atelocollagen type II based scaffold. They could show that their scaffolds did not cause any negative effects on bovine NP cells during a seven-day culture period. Compared to non-linked scaffolds, the cross-linked scaffold retained higher PG synthesis rate but lower elution of sulfated GAG into the surrounding medium. Moreover, when cross-linked, the scaffolds provided a more stable structure for the cells. O'Halloran *et al.* concluded that their enzymatically cross-linked, composite collagen-hyaluronan scaffold would show a high potential for developing an injectable cell-seeded scaffold to treat DD.

Regeneration of a given tissue is most effective, when transplanting cells from the same cell source. Regarding NP cells, their re-implantation leads to difficulties, since the autologous transplantation would require more cells than present in one healthy disc. Moreover, harvesting NP cells from healthy discs might lead to degeneration of the same. To address this problem, Yamamoto *et al.*[103] used a co-culture system with direct cell-to-cell contact with mesenchymal stem cells (MSCs). MSCs not only have the ability to differentiate in multiple cell types, but also serve as encouraging cells, insofar as they support the maintenance and proliferation for other cell types. Cells used for this experiment originated from New Zealand white rabbits. Yamamoto *et al.* could show that NP cells, having direct cell-to-cell contact, significantly increased their cell proliferation, DNA synthesis, and proteoglycan synthesis compared to conventional co-culture system or monolayer culture. Moreover, evaluations of supernatants revealed that growth factors associated with proliferation and cellular metabolism of nucleus pulposus cells were increased. Recently, there has been an increasing interest in using stem cells, since they are promising for treating a broad range of diseases and are suggested to have the potential to differentiate towards the phenotype expressed by nucleus pulposus cells of the IVD. In *in vivo* studies, Sakai *et al.* investigated the potential of MSC as an alternative cell source for NP cells.[104,105] They transplanted autologous MSCs labelled with the green fluorescent protein (GFP) and followed them for 48 weeks. During culture, increasing amounts of

GFP-positive cells where detected in the NP, indicating survival and proliferation of MSCs. Moreover, it has been documented that MSCs have immunosuppressive properties and are regarded to be non-immunogenic; this makes them particularly attractive for tissue engineering, cell and gene therapies.[106,107] Additionally, *in vivo* transplantation experiments revealed the ability of MSCs to migrate towards sites of injury, aiding wound healing and tissue repair;[108] a mobilization that is mainly promoted by cytokines/chemokines, adhesion molecules and proteolytic enzymes. .[109] Therefore, MSC might also migrate into damaged or diseased areas within the IVD. It has been shown that disc cells, as an early response, react to certain injurious stimuli by enhanced proliferation, metabolic activity.[104,110] and increased expression of proinflammatory mediators.[111,112] Next to further damage of the disc, those changes might also stimulate endogenous repair mechanisms, including recruitment of stem or progenitor cells.

2.10 Disc degeneration models

As stated in section 2.7, during the multifaceted process of disc degeneration, the breakdown of the extracellular matrix leads to structural deformation and dehydration of the intervertebral disc.[113] For simulating disc degeneration as well as regeneration strategies numerous *in vivo* and *in vitro* models have been developed. Larger animal models, including primates, porcine, cows and sheep have been widely used for studying the intervertebral disc (see also section 1.1).[114] The range for IVD research spans from *in vitro* cell culture approaches,[115] *in situ* organ culture[50] until *in vivo* animal experiments[116] and *ex vivo* human cadaver studies.[117] In *in vitro* cell culture, specific parameters can be investigated, but removal from the native tissue doubtlessly affects the cell behavior.[118,119] Whereas in *in vivo* experiments, the whole organism responds to the treatment. In consequence, factors, stimulated by the intervention, are difficult to control. Therefore, it is difficult to exclude, that other pathways cover the impact of a specific treatment. Studies with human IVDs generally are difficult to

accomplish since the material is difficult to obtain. Therefore, explant models of larger animals that are similar to human discs, are a good alternative, because controlled *in vitro* conditions can be applied to cells that are kept in their native surrounding. Lately, several explant models have been developed with different approaches and species.[5,36,120,121]

In the following chapter, the features of our newly developed bioreactor system will be described in detail.

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Chapter 3

Study design

3.1 Whole IVD organ culture bioreactor

To investigate IVD cells *in vitro* but simultaneously in their native environment, it is necessary to culture the entire organ in defined system. Such bioreactors should provide the possibility to maintain the composition, cell viability and homeostasis of the intervertebral disc by keeping the IVD under as physiological conditions as possible. This includes the application of the appropriate stress to avoid swelling of the tissue as well as the simulation of the daily loading pattern to assure the nutritional and metabolic transport throughout the disc. Many steps have been made until the final set up of our bioreactor was achieved. The current bioreactor was designed for application of uniaxial loading with frequencies up to 10 Hz. To withstand the immense forces, the bioreactor consists of a combination of polycarbonate and titanium alloy parts (Figure 7).

The disc chamber and the closing ring are made of polycarbonate, the force is applied via a titanium piston. The latter had to resist high frequencies, while the inside was bored to allow the media outflow from the disc chamber. Neither polycarbonate nor stainless steel pistons could be used for the present work. For prior investigations, both materials had been used successfully. The polycarbonate piston was designed for uniaxial diurnal load of 0.2 and 0.8 MPa.[1] However, it could not withstand the simulated physiological frequency of 0.2 Hz, which was applied in the following study; consequently, a stainless steel piston was designed for application of dynamic loading.[2] In turn, stainless steel would have been too heavy to transmit the oscillation at high frequencies due to their high moment of inertia.

To avoid media leakage but guarantee gas exchange with the surrounding, the piston was sealed with the polycarbonate ring via a gas-permeable silicone membrane that was attached to the piston and a silicone ring located underneath the polycarbonate ring.

Adjacent to the media in- and out-flow, two porous plates (stainless steel, Ø 18 mm and 8 µm pore size, for the first two studies respectively 3 µm for the

third study) were located adjacent to the discs. The IVD was kept centered in the bioreactor by always maintaining a compressive load in order to ensure contact of bony EPs to the porous plates. For the first two studies, culture media was pumped through Tygon or silicone tubing (SC0017 and SC0041, Ismatec SA, Glattbrugg, Switzerland) at 150 µL/min by a peristaltic pump (IPC, ISM 932, Ismatec SA) through the lower porous plates. Media was pumped around the centered disc and back through the upper plate to ensure that all external surfaces of the EPs and disc were moistened in fresh media. This setup was changed for the third study; there, the media tubing were blocked, since there we aimed to preserve within the media factors that might have been released by the discs.

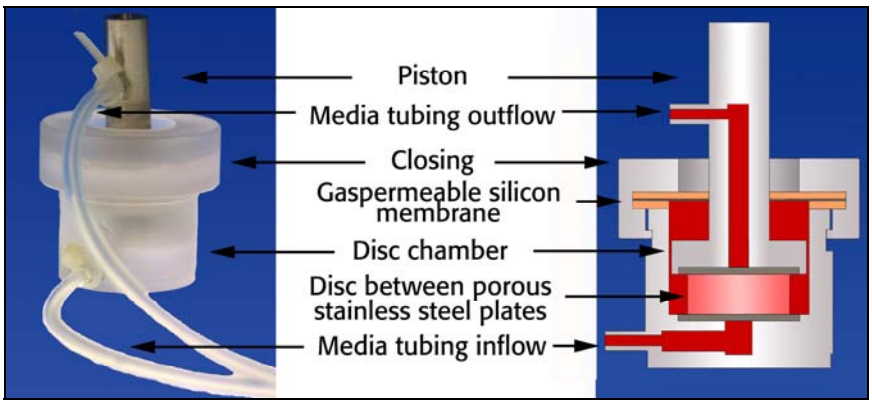

Figure 7: Polycarbonate bioreactor for culture of caudal discs under loading. IVDs with intact cartilaginous and bony endplates are maintained in a "sandwich" between two porous stainless steel plattens through which culture media is slowly refreshed (120 µl / min). Gas exchange is ensured by a thin permeable silicone membrane that seals the chamber from the top.

The bioreactor loading machine (Figure 8), consists of 4 units, with each bioreactor unit independently pneumatically actuated (dSpace data acquisition and control card, dSPACE GmbH, Paderborn, Germany; Matlab-Simulink, MathWorks, Inc., Natick, MA) under force control (load cell, Burster, Gernsbach, Germany, type 8432). The discs were cultured for 7 or 21 days under simulated physiologic loading, i.e., diurnal axial load (0.2/0.6 MPa, 8/16 hours) with cyclic

sinusoidal load during the 0.6 MPa active-phase (0.2/10 Hz ±0.2 MPa, 2x4 hours, Figure 8). The entire system was maintained in an incubator at 37°C, 5% CO_2, 90% humidity and ambient O_2.

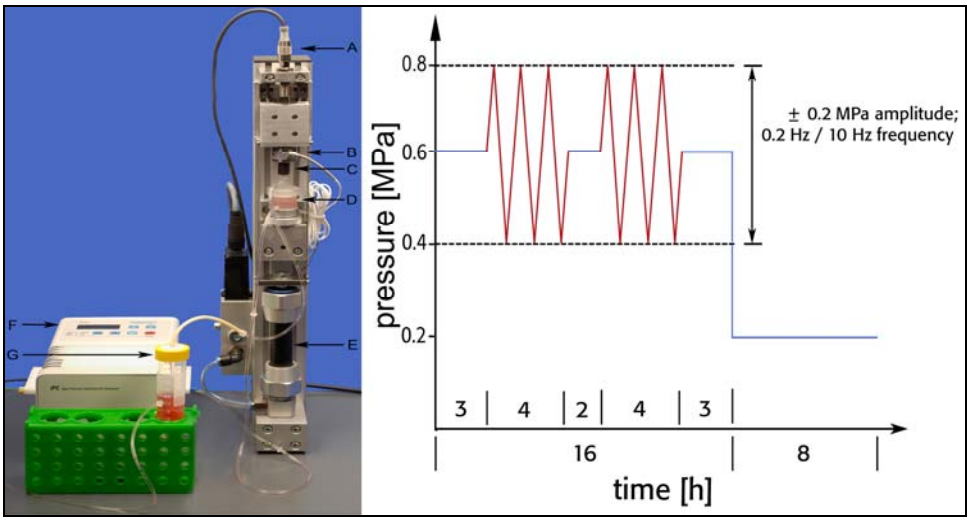

Figure 8: left: Bioreactor system for culturing IVD. A displacement sensor; B force sensor; C coupling; D polycarbonate bioreactor; E pneumatic actuator "fluidic muscle"; F peristaltic pump; G media reservoir. Right: loading scheme for culturing discs under diurnal axial loading; One cycle lasted 24 h: 16 h of 0.6 MPa (including 2 x 4 h sinusoidal cyclic compression) followed by 8 h of 0.2 MPa.

3.2 Disc harvest from ovine or bovine tail

3.2.1 Ovine IVDs

For the degeneration model, ovine discs from skeletally mature (age 2.5–5 years) Swiss alpine sheep (Ovis aries) were used; sheep were already being sacrificed for other experiments in our institution. All pre-euthanization procedures were approved by the Animal Experimentation Commission of the Veterinarian Office of the Canton of Grison, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Sheep were sedated with 0.3 mL Detomidin intramuscularly. Five minutes prior euthanasia, 25,000 IU of heparin was injected intravenously.

The animal was sacrificed via an overdose of pentobarbital (100 mg/kg). To avoid the formation of blood clots in the EPs of the tail discs, another 50,000 IU heparin was infused. Thereafter, the right and left arteria femoralis were ligated with a vascular clip proximal to the origin of the arteria femoralis profundus (Figure 9a). Subsequently, the abdominal aorta distal to the outlets of the arteriae renales and proximal to the iliac bifurcation was cannulated (Figure 9b). Fifty-thousand IU of heparin, dissolved in 1,000 mL Ringer's lactate solution, were infused into the abdominal aorta under a constant pressure of 100 mm Hg.

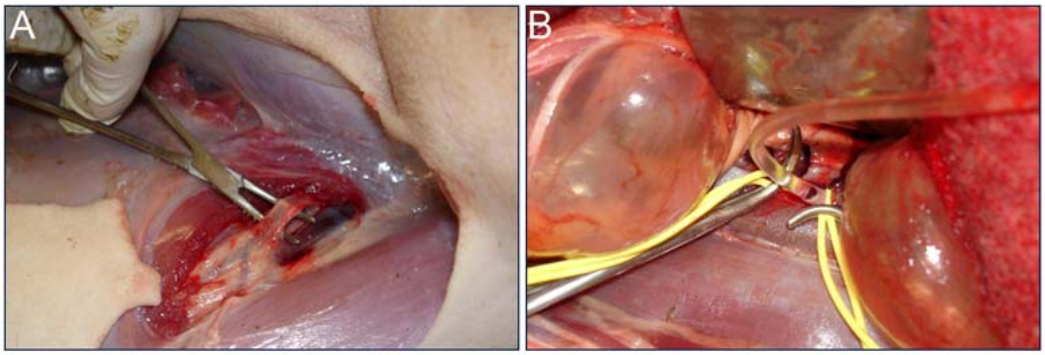

Figure 9: Ligation of the **A** ateria femoralis and **B** abdominal aorta.

Finally, the tail was amputated at the sacrum and freshly dissected under a laminar flow to prevent contamination. Tails were disinfected by BetadineTM solution prior to dissection and frequently sprinkled with PBS to prevent dehydration. The soft tissue was removed from the caudal spine to ensure easy localization of the IVDs. Spinous and transverse processes of the vertebrae were removed using bone removal pliers. After removing the surrounding tissue, blunt dissection was performed to remove muscles, nerves and blood vessels from the outer annulus and the vertebrae, without cutting into the disc (Figure 10).

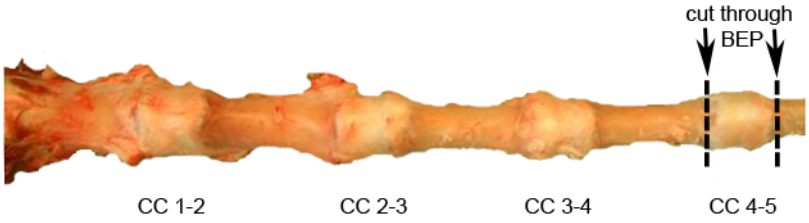

Figure 10: ovine caudal spine after removing the spinous and transverse processes. The first caudal IVDs (CC1-2; CC2-3; CC3-4; CC4-5) were prepared from each tail. Arrows mark the cutting zone through the bony end plates (BEP)

Four discs were prepared by making two parallel transverse cuts, through the bony EP just proximal and distal to the cartilaginous EP, with a histology band saw (model 30/833, Exakt Apparatebau, GmbH, Norderstedt Germany). The average maximum thickness of the convex bony EPs was 2.6 ± 0.7 mm. The ovine caudal discs were elliptical with major diameter of 10.5 ± 1.2 mm and minor diameter of 9.5 ± 1.2 mm. Disc heights were ~ 8 mm. Any debris on the cut surfaces were aspirated with a Pasteur pipette and surfaces were washed with a firm jet of phosphate buffered saline (PBS) using a syringe with an 18-gauge needle. Finally, discs were rinsed for 10 minutes in PBS containing 10% penicillin-streptomycin, washed in PBS and loaded in bioreactors with one disc each.

3.2.2 Bovine IVDs

For the regeneration experiments bovine tails were used, that were obtained from the local abattoir within 3 hours of death. Due to the experimental set up, bovine IVDs were cultured without EP. Similar to the ovine tail preparation, tails were disinfected by BetadineTM solution prior to dissection and soft tissue was removed. Instead of cutting through the bony EP, discs were removed by cutting along the proximal and distal disc-cartilage EP boundaries

using a sharp scalpel blade. Afterwards, discs were washed for 10 minutes in PBS containing 10% penicillin-streptomycin and rinsed in PBS before loading in bioreactors with one disc each.

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Effect of Limited Nutrition on Intervertebral Discs

Svenja Jünger, MSc[a], Benjamin Gantenbein-Ritter, PhD[a, d], Patrick Lezuo, Dipl-Ing[a], Mauro Alini, PhD[a], Stephen J. Ferguson, PhD[b] and Keita, Ito, MD, ScD[a, c]

[a]*AO Research Institute, Davos, Switzerland*
[b]*ARTORG Center, University of Bern, Switzerland*
[c]*Department of Biomedical Engineering, Eindhoven University of Technology, The Netherlands*
[d]*ARTORG Center, University of Bern, Switzerland*

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4.1 Summary

Study Design. Whole ovine caudal intervertebral discs (IVD) were cultured in sufficient and limited nutrition under simulated-physiological loading for seven and 21 days.

Objective. To study the effect of limited nutrition on disc cells embedded in their native tissue in short- and mid-term whole organ disc culture.

Summary of Background data. Nutrient-limited induction of disc cell death *in vitro* has been demonstrated and is believed to be a factor in disc degeneration. Nutrient-limited cell death and its consequences, as it relates to degeneration, has not been investigated in the intact IVD.

Methods. Ovine IVDs with endplates were cultured for seven and 21 days under simulated-physiological loading, either in media with limited (2 g/l) or sufficient (4.5 g/l) glucose concentration. Cell viability, relative gene expression, newly synthesized chondroitin sulphate (CS) content and metalloproteinase (MMP) activity were measured after culture and compared to fresh tissue.

Results. In sufficient glucose media, cell viability was maintained through seven days to 21 days of culture. In limited glucose, it dropped significantly to 62% in the annulus fibrosus (AF) and to 56% in the nucleus pulposus (NP) after seven days and remained so until 21 days (63% in the AF and 52% in the NP). No significant differences were found between culture conditions for relative gene expression, newly synthesized CS and inactive and active forms of MMP 13 and MMP 7.

Conclusion. With this culture system, whole IVD explants could be maintained up to 21 days. Cell viability decreased to 50-60% under limited nutrition within days and remained so up to three weeks. The surviving cells did not compensate matrix production in this time frame

4.2 Introduction

Degeneration of the intervertebral disc is generally believed to play a major role in pathogenic mechanisms causing low back pain,[1] which in one of the most common health problems for young to middle aged individuals in western society.[2,3] The underlying cause of disc degeneration is multifactorial. Genetic factors[4] and aging[5] are known predisposing factors for disc degeneration, but mechanical loading has also been shown to play a role.[6-8] The disc is the largest avascular organ of the body.[9] Because the main vascular supply is the capillary buds ending in the adjacent osseous vertebral endplates (EPs),[10,11] the cells in the disc must rely on diffusive and convective transport of nutrients and metabolites across a rather large distance.[12,13] As this nutrient/metabolite transport situation for the disc cells is already so precarious, any further limitation is believed to result in even lower density of viable cells responsible for maintaining a functional matrix. Conditions such as smoking, vascular disease, and vascular deficiency[5,14] have been associated with higher incidence of disc degeneration and calcifications in the cartilaginous EPs[15] occluding the osseous EP openings have been correlated with severity of disc degeneration.[16]

Several *in vivo* and *in vitro* models have been developed that could be used to investigate the effect of limited nutrition on disc degeneration. In dogs, Hutton *et al.* injected bone cement into the vertebrae adjacent to the EPs[17] and similarly Krebs *et al.* used vertebroplasties in adjacent vertebrae in the ovine lumbar spine. Although the changes in the disc were modest or absent, neither perfusion disturbance nor solute concentrations were measured, indicating the inherent difficulty to control solute transport in vivo. Alternatively, Horner *et al*. cultured bovine nucleus pulposus (NP) cells in an agarose gel diffusion chamber, comparing different cell densities, oxygen and glucose concentrations.[18] Such a system allowed precise relationships between solute concentrations and their effect on cell viability to be elucidated. However, in these studies NP cells were removed from their native complex three-dimensional environment and

underwent cell passaging. The possible effects of these conditions on cell behavior and their response to artificial stimuli are unknown.[19]

An alternative *in vitro* method is tissue culturing, i.e. leaving the cells in their native environment.[10,20,21] However, the difficulty with tissue culturing has been maintenance of the high osmotic potential of the tissue as well as mimicking the complex physiological loading conditions of the tissue. Recently, a novel *in vitro* system was developed for culturing whole intervertebral disc (IVD) explants with natural controlled loading through intact EPs.[22] Cultured with EPs, the biomechanical integrity of the disc can be maintained by anchoring the annulus fibrosus (AF) fibers and preventing dislocation of the NP. Thus, the disc cells remain viable in their native extra cellular matrix (ECM) and culturing effects caused by artificial environment or passaging is avoided. In this study, the effect of nutritional insufficiency on cell viability was measured in the whole disc organ culture system and the anabolic and catabolic response of the remaining cells was investigated. We specifically tested the hypothesis that with reduced nutrition a new equilibrium of viable cells and matrix turnover would occur.

4.3 Materials and Methods

The procedure for disc harvesting and preparation, as well as the bioreactor used in this study, were the same as described previously.[22] For this study, discs were harvested from 15 skeletally mature (3 – 5 years-old) Swiss alpine sheep (*Ovis aries*), which were already being killed for other experiments in our institution. All pre-euthanization procedures were approved by the Animal Experimentation Commission of the Veterinarian Office of the Canton of Grison, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Five or six caudal discs were prepared from each sheep tail. Due to conventional tail amputation in lambs, six discs were not always available. To exclude the possibility of disc level dependent cell

metabolism rates[23], the nutritionally sufficient and limited as well as day 0 (d0) control discs were distributed among the harvested caudal levels.

Four bioreactor chambers (Figure 11D) were loaded with one disc each and the remaining discs were used as fresh d0 controls.[22] The bioreactor system (Figure 11), consists of four units, with each bioreactor unit independently pneumatically actuated (dSpace data acquisition and control card, dSPACE GmbH, Paderborn, Germany; Matlab-Simulink, MathWorks, Inc., Natick, MA USA) under force control (load cell, Burster, Gernsbach, Germany, type 8432). The discs were cultured for seven or 21 days (d7 or d21) under simulated physiological loading, i.e. diurnal axial load (0.2/0.6 MPa, 8/16 h) with cyclic sinusoidal load during the 0.6 MPa active-phase (0.2 Hz ± 0.2 MPa, 2x4 h, Figure 12). The entire system was maintained in an incubator at 37°C, 5% CO_2 and ambient O_2. To select an appropriate "nutritional stress", pilot experiments were conducted, with six experimental and six sufficiently supplied (4.5 g/l glucose) discs (from three sheep) cultured for seven days. Experimental discs were cultured in three different glucose concentrations, i.e., 3.0, 2.0 or 1.0 g/l (n = 2 per group). Previously 4.5 g/l was used and demonstrated to maintain cell viability.[22] Media were prepared by adding appropriate amounts of glucose (SIGMA-Aldrich, Buchs, Switzerland) to DMEM base, 25 mmol/l HEPES buffer, 3 ml/l gentamycin, 0.58 g/l L-glutamin, 3.7 g/l $NaHCO_3$, 0.11 g/l sodium pyruvate, 0.015 g/l phenol red, 1% P/S, 10% FCS (all from SIGMA-Aldrich). These pilot experiments revealed that a glucose concentration of 2.0 g/l (= "limited nutrition condition") was found to induce about 50% cell death. Hence, in the main series, discs were cultured for seven and 21 days either under sufficient (n = 12 for d7, n = 4 for d21) or limited (n = 17 for d7, n = 4 for d21) nutrition. 50 ml of media was circulated continuously through a peristaltic pump (Ismatec, Glattbrugg, Switzerland) at a flow rate of 120 µl/min and exchanged every 2-3 days. The cultured discs were compared to 11 freshly harvested discs (= d0 control) in the day 7 and to 6 fresh discs in the 21 day culture. Overall the discs originated from nine sheep for the day 7 and from three sheep for the 21 day culturing experiment.

Since the difference of osmolality between sufficient and limited media was only minor (6.5mOsm/kg H_2O, measured in DMEM w/o FCS), osmolarity of the media was not adjusted, avoiding possible interfering effects from substitutes. After culture, EPs of discs were removed with a scalpel blade and the AF and NP were separated with a biopsy punch (diameter = 5 mm). Tissues were then cut into three equal parts: one third was used to assess cell viability; the second third was used to assess gene expression; and the last third was further split for MMP zymography and determination of newly synthesized aggrecan content.

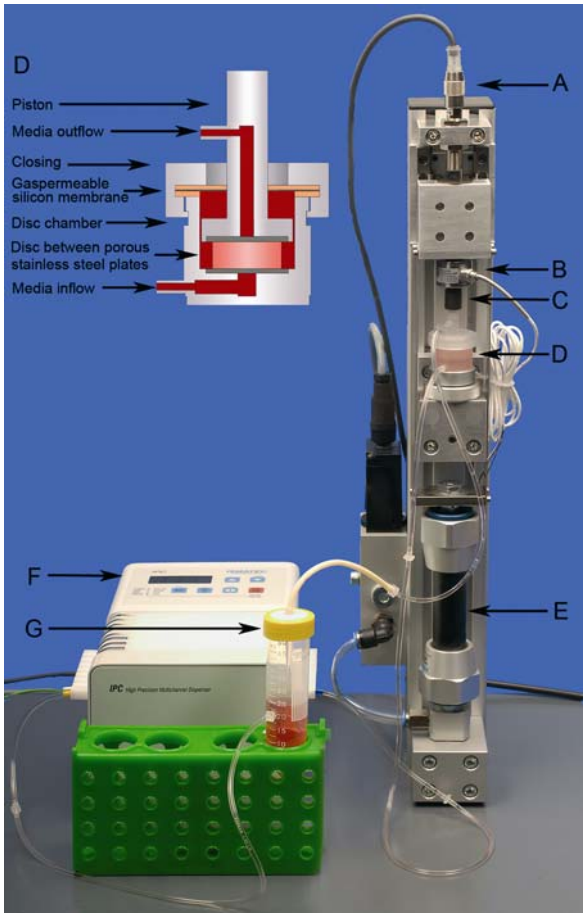

Figure 11: Bioreactor system for culturing ovine IVD. A displacement sensor; B load sensor; C coupling; D schematic diagram of polycarbonate bioreactor; E pneumatic actuator "fluidic muscle"; F peristaltic pump; G media reservoir.

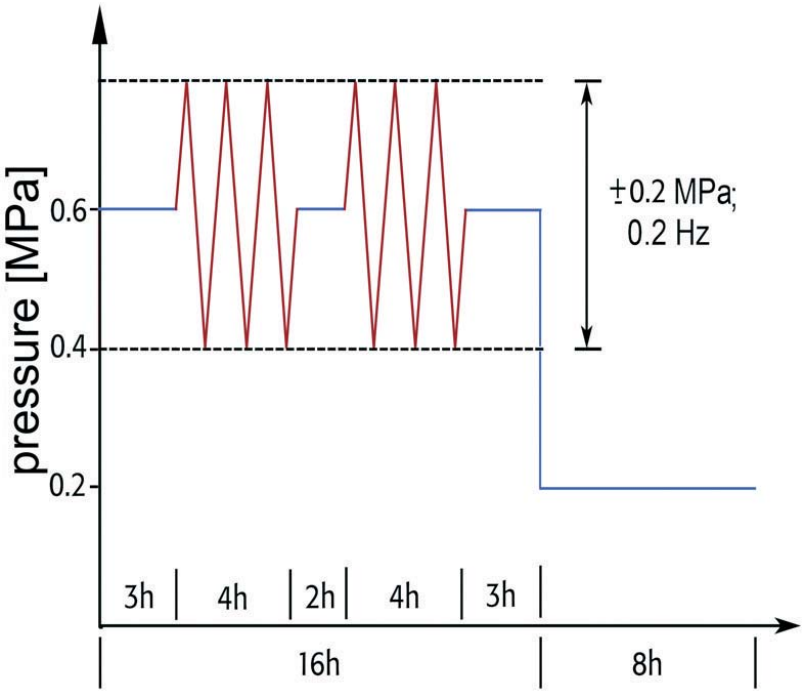

Figure 12: Loading scheme for culturing discs under simulated physiological axial loading; One cycle lasted 24 h: 16 h of 0.6 MPa (including 2 x 4 h sinusoidal cyclic compression) followed by 8 h of 0.2 MPa.

Cell viability was determined with the LIVE/DEAD staining kit (Molecular Probes, Leiden, The Netherlands). Disc tissue was incubated in serum free medium supplemented with 10 µM calcein AM green and 1 µM ethidium homodimer-1 under free swelling condition for 2 h. Stained samples were visualized on an inverted confocal laser scanning microscope (LSM510, Zeiss, Jena, Germany). CLSM stacks were imaged with 50% image overlap at 5.7 µm image intervals with a pin-hole diameter of 1 Airy Unit. Stacks were split into single color channel grey scale 8-bit images and single images in the stack (512 x 512 pixels) were quantified using a custom-made macro[24] in ImageJ software (http://rsb.info.nih.gov/ij/). The macro ("binary-Otsu-counter") consists of a thresholding step that passes a binary image to the plug-in "nucleus counter" (available as a plug-in bundle from the McMaster University, Biophotonics

Facility, http://www.macbiophotonics.ca/imagej), which then uses the "Otsu" method for particle counting. The range of pixels used for the initial thresholding for the conversion into a binary image was set from 100 to 255 for the red and from 75 to 255 for the green channel. The minimum and maximum island size on the red channel was set to 5-50 and for the green channel to 10-100 pixels. The macro has been written to run each channel separately and then to combine the outputs at the end in a spread-sheet based program (Excel, Microsoft Inc., Redmond, WA, USA). The cell viability was then estimated on a subset of ten consecutive images in the stack. The first image was chosen as that with 50% of the maximum cell number per image in the stack plus 5 images deeper into the tissue to prevent artifacts from the cut surface.

To assess gene expression, disc tissue samples were flash frozen in liquid N_2 and pulverized into powder. Two ml Eppendorf tubes containing a free 8 mm stainless-steel ball and a counterpart (placed in the lid) were pre-cooled for 10 min in liquid N_2. Then the tissue was added and cooled for another 30 s. For pulverization, samples were vibrated for 20 s at 25 Hz with a tissue lyser (Quiagen). If necessary, samples were again cooled in liquid N_2 and vibration repeated until samples were pulverized. Total RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), using a modified TRIspin method. Reverse transcription was performed as previously reported.[25] Relative gene expression of selected anabolic and catabolic genes (oligonucleotide primers, Microsynth, inc., Balgach, Switzerland, as described previously)[22] i.e. aggrecan (ACAN), collagen I (COL1A1), collagen II (COL2A1), ADAMTS4 and MMP13 were measured relative to the "housekeeping" gene ribosomal 18S[26] and quantified by real-time RT-PCR (ABI GeneAmp 7500, Applied Biosystems, Foster City, CA, USA)[25] Samples with undetectable RNA concentration for the target gene but with detectable 18s gene expression ($C_t < 18$) were assigned at C_t of 45 (maximum number of cycles).

Newly synthesized aggrecan content in each tissue sample was assessed by measuring the amount of chondroitin sulphate 846 (CS846) epitope of the

proteoglycan aggrecan. This monoclonal antibody recognizes a chondroitin sulfate epitope on the largest aggrecan molecules showing 100% aggregate ability with hyaluronan. The epitope content is barely detectable in normal adult cartilage and discs (low metabolic turnover), is increased in osteoarthritic cartilage and degenerated discs (repair attempt), and is highest in fetal and neonatal cartilage (high metabolic activity). [27] In vitro studies have shown a direct correlation of the content of this epitope in cartilage to aggrecan synthesis rate, measured by incorporation of [35 S] sulfate into the glycosaminoglycans (GAG) of aggrecan,[28,29] and its half-life has been estimated to be 15-20 hr.[30] Therefore, the 846 epitope may be considered to be representative of a newly synthesized subpopulation of aggrecan molecules at any point in time.[31] Each disc tissue sample was lyophilized, flash frozen in liquid N_2 and pulverized into powder (as described above), incubated at 4°C for 48 h with agitation in 1 ml of CHAPS buffer (4 M guanidinium chloride in 50 mM sodium acetate, 0.5% CHAPS (3-(3-cholamidopropyl) diethyl-ammonio-1 propanesulfonate), pH 5.8, with protease inhibitor cocktail, Sigma P8340) to extract the proteoglycans. The extract was dialyzed against 50 mM sodium acetate pH 6.3. To change the buffer system, aliquots of 50 µl were lyophilized over night and dissolved in buffer supplied with the CS846 Epitope ELISA kit (IBEX, Montréal, Canada). Samples were treated according to the manufacturer's instructions, and concentration of CS846 was measured and normalized to number of living cells using the following formula:

$$\frac{CS846}{cell} = \frac{CS}{W} * p * \frac{V}{C}$$

Where CS = amount of CS846 in µg, W = wet weight of the sample in mg, p = density of the tissue, 1 / kg, V = volume of the imaged tissue in μm^3 and C = estimated number of living cells from LIVE/DEAD staining per volume.

Determination of denatured and total collagen content was assessed by determination of the OH-proline content. [32] The denatured collagen was

solubilized with alpha-chymotrypsin and the remaining collagen chains were digested with proteinase K as previously described.[31,31]

For the matrix metalloproteinase (MMP)-zymogram, tissue samples were flash-frozen in liquid N_2 and pulverized (as described above). Pre-chilled extraction buffer (PBS + 0.5% Triton X-100 + 5 µl/ml protease inhibitor cocktail, (SIGMA) was added to the tissue, which was then homogenized, incubated on ice for 10 min and centrifuged at 14,000 g for 10 min. The total protein concentration was determined using the Bradford quick start assay kit (Biorad, Reinach, Switzerland). Approximately 60 µg total protein per lane was mixed with 2x loading buffer (0.1 M Trizma base, glycerol 87% (Fluka, Buchs, Switzerland) and 0.02% bromophenol blue (Biorad)) and loaded on the gel. The protein extracts were run on a two-phase gel system with stacking gel (4% acryl-amide (Biorad), Tris-HCl pH 6.8) and resolving gel (8% acryl-amide, 1% gelatine (Fluka), 1.5M Tris-HCl pH 8.8). Electrophoresis was run for 7.5 h where for the first 15 min it was run at 3V/cm and afterwards at 6V/cm. The gel was then incubated for 1 h in renaturation buffer (2.5% triton X-100 in ddH_2O) at RT, afterwards rinsed with H_2O, followed by overnight incubation in developing buffer (50mM Tris-HCl pH 8.0, 5mM $CaCl_2$ in H_2O) at 37°C. The next day, the gel was stained with Coomassie blue (2.5 g Coomassie blue R-250, 100 ml acetic acid, 250 ml isopropanol in 1L ddH_2O) for 2 h and finally destained with destain buffer (10% Methanol, 10% Actetic Acid in ddH_2O). Imaging was done with Gel-Doc 2000 (Biorad), and ImageJ software (http://rsb.info.nih.gov/ij/) was used for quantification.

For statistical analysis, all data were normalized to the mean fresh d0 control levels and a Wilcoxon signed rank test blocked for sheep was used for cell viability, RT-PCR and CS846 content (SPSS Inc., Chicago, IL, USA). Relative Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. ΔC_t data was transformed and $\Delta\Delta C_t$ values averaged within sheep and culture condition. For all statistical analyses, $p < 0.05$ was considered significant.

4.4 Results

Cell viability in fresh d0 discs was ~90% in both annulus fibrosus (AF) and nucleus pulposus (NP) (AF = 92%, 95-89%; NP = 93%, 95-87% (median, IQR)). For discs cultured in sufficient media for both culturing periods (d7 and d21 sufficient), no significant differences in cell viability were detected (d7: AF = 91%, 91-86%; NP = 81%, 86-79%; d21: AF = 88%, 79-94%; NP = 87%, 82-94%). Cell viability dropped significantly to 62%, 66-43% in the AF and 56%, 58-37% in the NP in the limited glucose group after seven days (d7 limited) (AF, p = 0.02; NP, p = 0.03; Figure 3). In limited nutrition, cell viability stabilized thereafter and was similar up to 21 days (AF = 63%, 51-66%; NP = 52%, 40-63%;Figure 13).

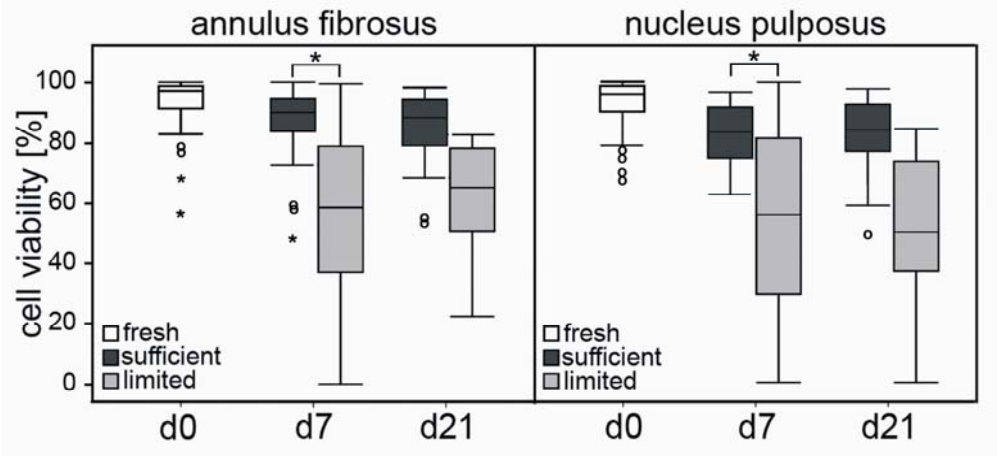

Figure 13: Cell viability in annulus fibrosus (left) and nucleus pulposus (right) of fresh discs (d0) and discs after seven and 21 days of culture (* is p< 0.05)

Gene expression did not significantly differ between discs cultured in sufficient and limited media for both tissue types (AF or NP) after one week. Anabolic genes, e.g. aggrecan (ACAN) and collagen II (COL2A1), tended to be up-regulated, relative to d0, especially in the NP, whereas ADAMTS4 and MMP13 tended to be down-regulated in both tissues at the end of culture, but this was not statistically significant (Figure 14A). Even after three weeks cultivation, no significant differences between sufficient and limited cultured discs were

observed. Anabolic gene expression pattern after d21 culture was similar to that at d0. In contrast, the catabolic gene ADAMTS4 tended to be up regulated at d21 in both tissues, whereas MMP13 showed a tendency for up regulation only in AF and tended to be down regulated in NP (Figure 14B).

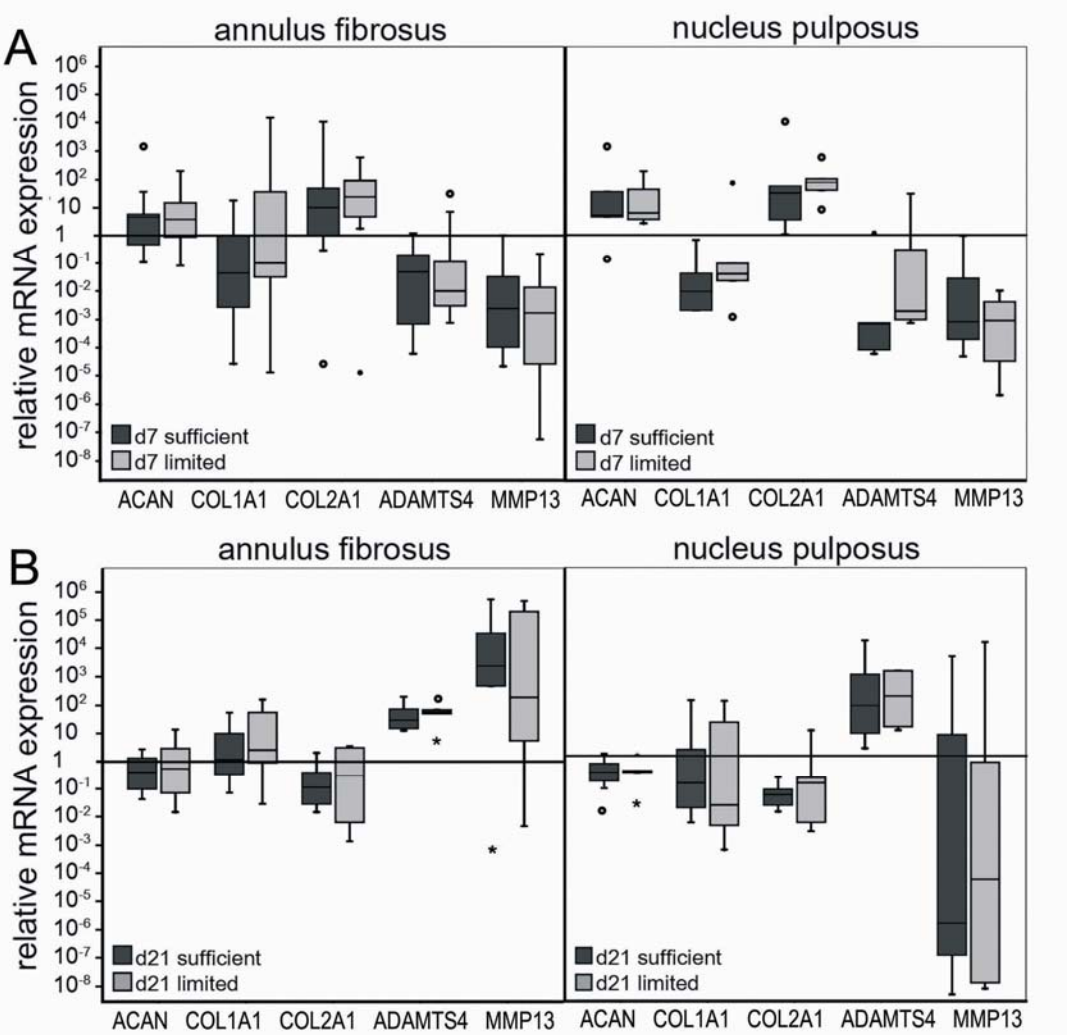

Figure 14: Relative gene expression of three anabolic and two catabolic genes after 7 days of culture (A) and 21 days of culture (B); Note that anabolic genes such as aggrecan (ACAN) and collagen II (COL2A1) are found slightly up regulated and ADAMTS 4 and MMP 13 were found down regulated at the end of seven days of culture.

Newly synthesized aggrecan content was not significantly different between culture conditions, for both AF and NP after seven or after 21 days (Figure 15). However, after d21, a slight increase of CS846 synthesis was observed in the limited group for both AF and NP, even though, due to the low sample size, this was not statistically significant (AF: sufficient = 0.023 µg/cell, 0.009-0.028 µg/cell, limited = 0.041 µg/cell, 0.019-0.105 µg/cell, p = 0.23; NP: sufficient = 0.128 µg/cell 0.066-0.454 µg/cell, limited = 0.1547, 0.1177-0.3087 µg/cell, p = 0.14; values are medians, ± range).

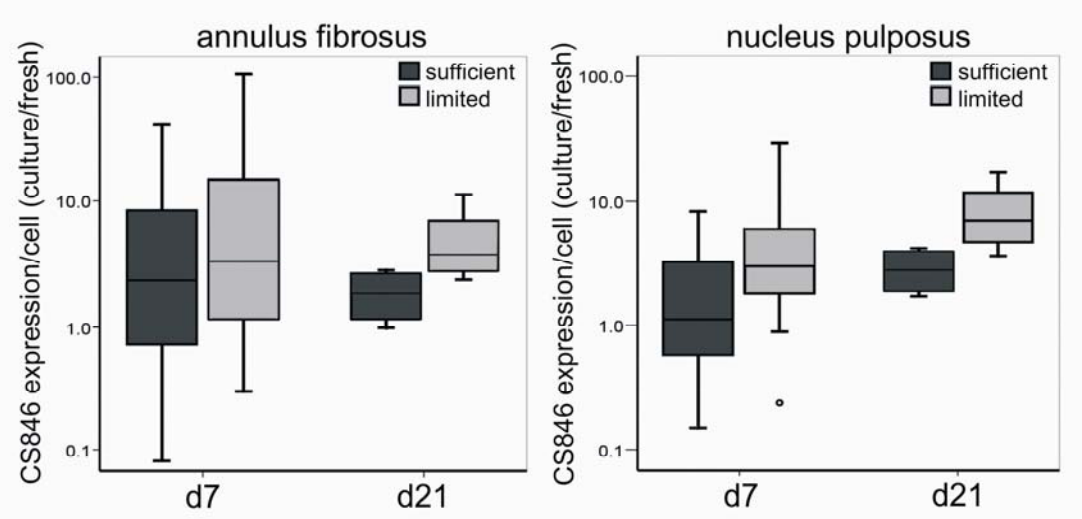

Figure 15: CS846 content in the annulus fibrosus (left) and nucleus pulposus (right) of discs cultured for seven or 21 days (* is p< 0.05); fresh = d0 fresh control; sufficient = sufficient glucose (4.5 g/l) limited = limited glucose (2 g/l).

No denatured collagen could be detected at d0 or d21 (data not shown). For MMP activity after 7 days, mainly MMP7 (pro-active and active forms) was found expressed in AF and NP in all discs, whereas pro- and active MMP13 were detected in only some discs (Figure 16). For the AF, under limited nutrition a slight increase of active MMP7 was measured relative to d0, however, this increase was not significant (p = 0.07). For pro-MMP13 (inactive form), only nine out of 40 (AF) and eleven out of 35 (NP) discs tested positively in zymography.

Activated MMP13 was detected very seldom, just in four (AF) and in one (NP) disc. Increasing cultivation time did not change the MMP expression pattern up to 21 days (data not shown).

4.5 Discussion

This study utilizes a previously established *in vitro* whole disc organ culture system using discs from a large animal model.[22] To investigate the responsiveness of disc cells to different biophysical and biomechanical stimuli in their native ECM *in vitro,* it is indispensable to culture the entire organ, i.e., the IVD composing of AF, NP and endplates (EP).[20,33-37] Maintaining the anchorage of the annular collagen lamellae in the EPs allows application of mechanical loads in a natural fashion, where nuclear pressure is balanced by annular stresses without excessive radial bulging,[38] the natural fluid exchange of the disc is maintained and biochemistry is not altered through loss of molecules via the newly created free surfaces.[15] Recently, a variety of culture systems have been reported that keep the endplates attached. However, almost all of these are IVDs from small animals such as rats and rabbits. Especially in cases where younger animals were included, the cell population differed with inclusion of notochordal cells, whose effects are not yet fully understood.[39,40]

Furthermore, disc dimensions and diffusion lengths of small animal models are not comparable to the human case.[10,41,42] Lee *et al.* evaluated the feasibility of culturing bovine caudal discs with endplates and reported a drastically reduced cell viability compared to discs without endplates.[43] For organ culturing of large animal IVDs, we have demonstrated that blockage of the main nutrition pathway of caudal discs by blood clotting in the capillary buds in the EPs can be avoided by systemic anticoagulation.[22] This may also be achieved if the transport routes of the EPs of the discs are opened by weak acidic treatment or jet-spraying cleaning techniques within hours post-mortem in discs obtained from the local abattoir.[44]

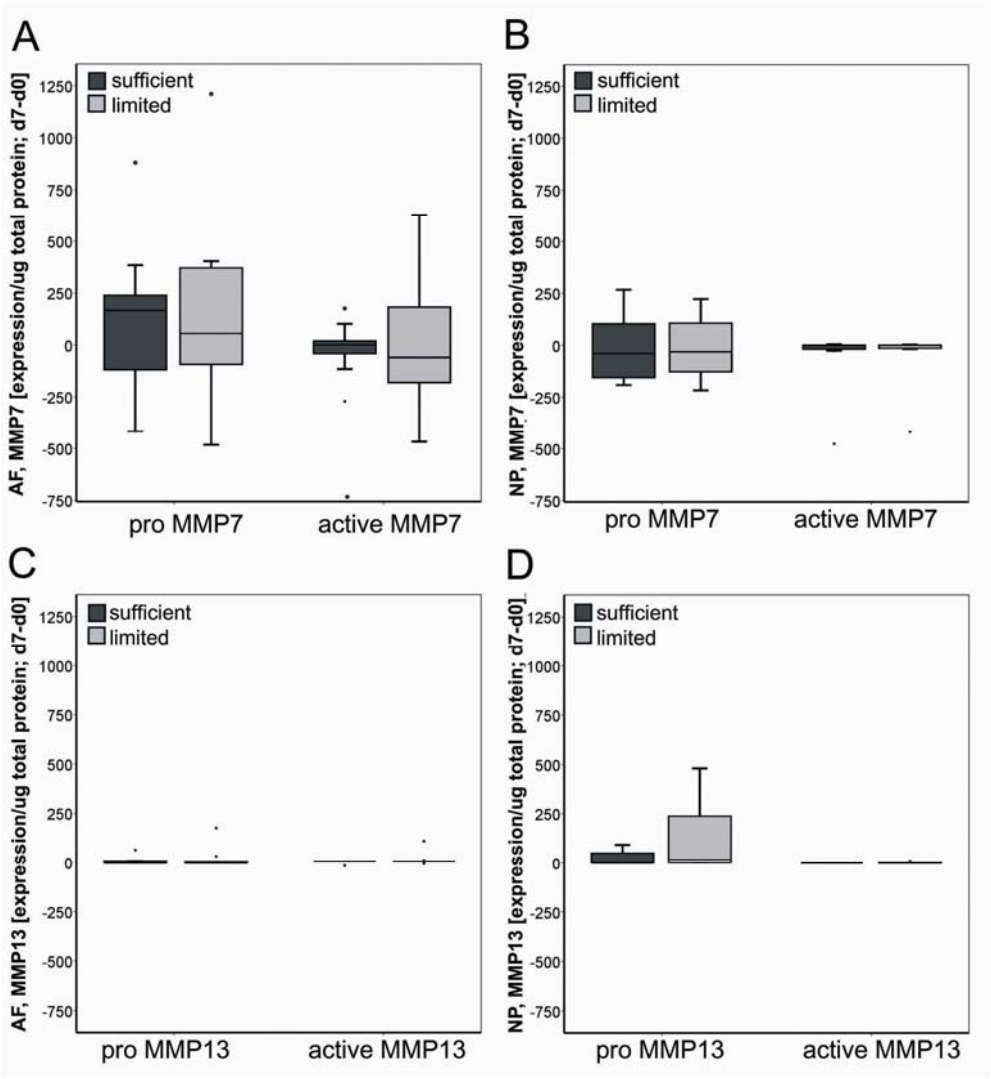

Figure 16: Pro and active forms of MMP 7 (A+B) and MMP 13 (C+D) expression in the annulus fibrosus (A+C) and nucleus pulposus (B+D) of discs cultured for seven days; sufficient = d7 sufficient glucose (4.5 g/l) limited = limited glucose (2 g/l).

In this study, we present new data from our further improved culture system by applying simulated-physiological loads experienced by the IVD *in vivo* and also a three times longer culturing period was investigated. Gantenbein *et al.* applied diurnal loading to the IVDs in the bioreactor for seven days.[22] In the current study an additional low magnitude cyclic loading at 0.2 Hz was applied in addition to diurnal loading. Although this is not a direct controlled comparison,

relative to the previous study, cell viability of discs in sufficient media in both studies was similar but the variance between samples was even lower this time, in both NP and AF. Gene expression, relative to day0 controls, seemed more beneficial with simulated-physiological loading compared to simple diurnal loading, i.e. anabolic matrix protein mRNA expressions were up-regulated and catabolic protease mRNA expression was down-regulated.

The increased cell death (50-60%) caused by reducing the primary energy source, i.e. glucose, is in agreement with earlier work on the physiology of disc cells.[2,3] Our study is, to our knowledge, the first to confirm Horner & Urban's (2001) *in vitro* cell culture work on the effect of low glucose conditions on disc cells *in situ* embedded in their natural extra cellular matrix (ECM). Horner & Urban (2001) cultured primary disc cells under limiting glucose conditions in a diffusion chamber and induced cell death in a similar way. They found that cell viability initially dropped, dependent on distance from nutrient source, but then stabilized to a new equilibrium after 2-4 days of culture. In our system, a new equilibrium of cell viability was reached similarly within days and remained stable up to three weeks. In addition, we could observe dead cells as well as evidence for cell mitosis at day 0 but also still after 7 and 21 days. Assuming that the nuclei of dead cells are disintegrating after 2-3 days and DNA is broken down by DNAse these should not be detectable anymore by ethidiumhomodimer-1. Thus, it may be likely that there is a balanced cell renewal after up to 21 days in culture, under both culture conditions. (Figure 17). Horner & Urban also detected a dramatic decrease of cell viability towards the center of their chamber, which was also dependent on seeded cell density. However, compared to their culture conditions, our limited culture medium had double the glucose concentration and also contained almost double the concentration of FCS. Nevertheless, cell viability was observed to decrease throughout the whole disc, resulting in areas of mainly dead or viable cells. Although it would have been interesting to relate our cell viability results directly to Horner & Urban's set-up with respect to distance from nutrient source, i.e. the EPs for the NP and IA, this was not possible with our

cLSM staining/scanning protocol because once the organ was dissected and the tissues were incubated under free swelling, orientation of the tissue was difficult to re-establish. Remarkably, 1g/l glucose is the physiological concentration in the human blood whereas in our organ culture an about four times higher concentration of glucose was required to keep the cells alive during cultivation.

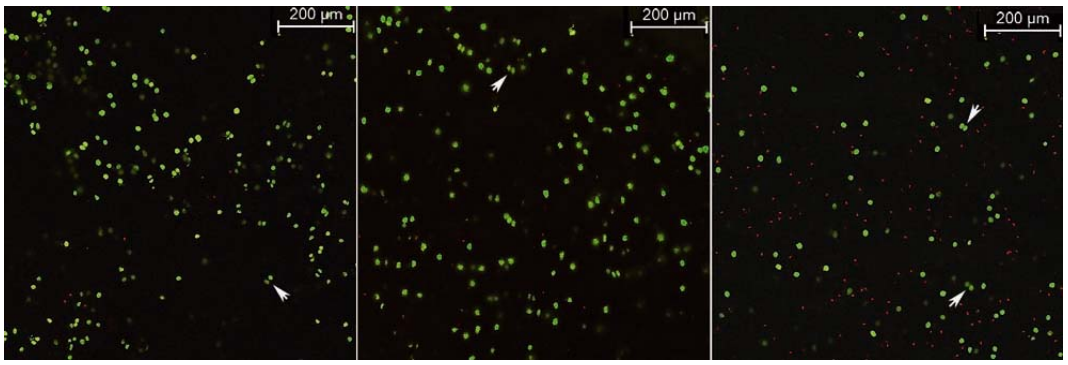

Figure 17: Representative images of Calcein AM / Ethidium homodimer staining of nucleus pulposus tissue of a fresh disc (left) and after a 21 day culture under sufficient (middle) or limited (right) conditions; green = living cell; red = dead cell; note the cell divisions (arrows).

This discrepancy may be attributed to two reasons. First, our procedure of systemic heparinization and vascular evacuation, that aimed to open nutrient transport into the disc, still did not result in equally diffusive endplates as compared to the *in vivo* situation. This reduced permeability may have been the cause for the need of a much higher glucose concentration in the surrounding media to ensure physiological levels of glucose in the center of the disc. Alternately, the cultured discs were kept under normoxic conditions rather than under hypoxic condition and oxygen tension inside the disc was not determined. An increased oxygen tension, on the other hand, could have caused an increase in glucose consumption of disc cells.[18] These physical and chemical parameters should be included in further development of the bioreactor system as they provide valuable information on disc metabolism.

It is generally believed that any kind of blockage of the nutrient supply, e.g. endplate injury and/or calcification, would cause a decrease in the density of disc cells which are necessary to maintain a functional matrix.[16] According to this generally accepted nutrition-induced disc degeneration mechanism,[45] a decrease in cell viability is hypothesized to stimulate a compensation by the remaining cells, resulting in increased matrix turnover but in overall balance a general catabolic breakdown of the matrix. However, no statistically significant changes in gene expression and MMP activity supporting early disc degeneration were observed even after three weeks cultivation, neither relative to fresh discs nor between sufficient and limited cultured discs. After three weeks culture, only the amount of newly synthesized aggrecan tended to be increased in the limited cultured discs, compared to the ones in sufficient nutrition in both AF and NP. The extra-cellular matrix of the NP contains mainly type II collagen and aggrecan. The CS846 epitope is present mainly in a newly synthesized high molecular weight aggrecan subpopulation.[46] With advanced disc degeneration, the collagen matrix is disrupted and the proteoglycan content decreased.[47] However, in early stages of degeneration, collagen and aggrecan synthesis have both been found to be elevated, relative to the healthy state.[3,31,46-49] Therefore, the slight increase of CS846 in our data might be interpreted as a rescue effort and early response of the disc.

Generally, the results from the semi-quantitative zymography did not show differences between limited and sufficient nutrition groups, or between fresh and cultured discs, after one or three weeks of cultivation. MMPs are secreted in latent form (pro-MMPs) and their activation is regulated by specific TIMPs (tissue inhibitors of metalloproteinases) which inhibit certain particular MMPs.[50] Le Maitre et al.[51] reported that MMP7 is more resistant to TIMP-1 and -2 and therefore might have a key function in terms of disc matrix remodeling. Consistent to their findings, in this study, after seven days, mainly MMP7 was detected whereras MMP13 was hardly evident in the discs. The low amount of

detectable MMP13 is in agreement with Le Maitre's findings, as it was nearly not expressed in non-degenerated discs.

4.6 Conclusion

We have presented a disc organ culture system with simulated-physiological loading of caudal ovine discs cultured with media containing sufficient and limited concentration of glucose. Although decreasing glucose concentration did cause substantial reduction in living cells, it was apparently not enough to provoke other indications for disc degeneration at the gene expression and protein level during a short and mid term whole organ culture. With approximately $2x10^6$ cells/g wet weight in fresh discs, cell density is relatively low. One could assume that this limits sufficient cell-to-cell signaling; in turn, the single cell is not aware that the neighbor cell is dying. If the direct environment of the cells would change, e.g. due to physiological stress, one could expect an earlier respond of the cells. To simulate this, in a next step, we will investigate the synergistic effects of limited nutrition combined with low (0.2Hz) and extreme high (10Hz) frequency loading.

4.7 Acknowledgement

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Effects of Limited Nutrition and High Frequency Load on Intervertebral Discs

Svenja Illien-Jünger, MSc[a], Benjamin Gantenbein-Ritter, PhD[a,b], Sibylle Grad, PhD[a], Patrick Lezuo, Dipl-Ing[a], Stephen J. Ferguson, PhD[b], Mauro Alini, PhD[a] and Keita, Ito, MD, ScD[a, c*]

[a]*AO Research Institute, Davos, Switzerland*
[b]*ARTORG Center for Biomedical Engineering Research, University of Bern, Switzerland*
[c]*Department of Biomedical Engineering, Eindhoven University of Technology, The Netherlands*

The content of this chapter is accepted in Spine" The Combined Effects of Limited Nutrition and High Frequency Loading on Intervertebral Discs with Endplates".

5.1 Summary

Study design. Whole ovine caudal intervertebral discs (IVD) were cultured under simulated-physiological or high frequency loading and either sufficient or limited nutrition for seven days.

Objective. To study the effect of high frequency loading under sufficient or limited glucose conditions and investigate additive effects of load and nutrition on cell survival, gene expression and cell activity after seven days of culture.

Summary of background data. Limited nutrition and certain mechanical stimuli are generally believed to be etiological factors for disc degeneration. Although these effects and their interactions have been demonstrated in cell culture, no investigations have been reported in entire discs.

Methods. Discs were maintained in a whole organ culture bioreactor system under simulated-physiological (0.2 Hz) or high frequency (10 Hz) loading, either in media with limited (2 g/l) or sufficient (4.5 g/l) glucose concentration. After seven days, cell viability, relative gene expression, newly synthesized chondroitin sulphate (CS) content, glycosaminoglycan (GAG) synthesis rate and disc morphology were assessed after culture and compared to fresh tissue:

Results. Culture under either limited glucose or high frequency loading conditions led to a significant drop in cell viability. Combined treatment with limited glucose and high frequency loading resulted in an additive increase in cell death in both the annulus fibrosus and nucleus pulposus and in an increase in MMP13 gene expression.

Conclusion. Supporting in vivo studies and cell culture experiments, high frequency loading simulating vibration conditions shows detrimental effects on IVD cells in whole organ culture. The effect on cell viability was exacerbated by limited nutrition culture. However, neither frequency nor limited glucose affected cell metabolism, measured by GAG synthesis rate. Longer culture periods may be required to detect changes at the extracellular matrix level.

5.2 Introduction

Low back pain (LBP) is the primary cause of disability in the active age group in Western society, with a lifetime prevalence estimated at 80%.[1,2] In many cases, low back pain is related to intervertebral disc (IVD) degeneration.[3] Although its etiology is still unclear, a potential cause for degenerative changes is diminished nutrition.[4] The disc consists of soft tissue between the bony vertebrae. The cartilaginous endplates (EPs) are adjacent to the gelatinous nucleus pulposus (NP) that is surrounded by the annulus fibrosus (AF), a highly orientated fibrous tissue with a lamellar structure. As the largest avascular structure in the human body,[5] the IVD highly depends on diffusion of nutrients into the disc center. Because of low cell density (4'000-9'000 cells/mm^3)[6] , low cell activity and avascular condition, the disc has limited self-repair capabilities. As early as 1931, Beadle observed that nutrition to the disc is mainly supplied via the capillary vascular buds in endplates (EPs)[7] and more recent studies have corroborated this finding.[8,9] During a lifetime, the EPs become less permeable correlating with an increase of disc degeneration, which is believed to be due to an even further decrease in nutrient supply.[10] Conditions such as smoking, vascular disease, and vascular insufficiency have been associated with higher incidence of disc degeneration and calcification in the cartilaginous EPs.[11,12,13] Moreover, occluding the osseous EP openings have been correlated with severity of disc degeneration.[14]

There are also indications that dynamic loading can be deleterious to the disc. Dynamic compressive loading has been shown to lead to degenerative changes to the disc, such as breakdown of the extra cellular matrix (ECM).[15,16] In epidemiological studies exposure to whole body vibration (WBV) has also been related to low back pain (LBP). Particularly vibration in the 4 to 10 Hz range was found to stimulate degenerative processes.[17,18] This may be due to resonance. The natural vibration frequency of the human body ranges from 3 to 14 Hz,[19] with the higher frequencies representing bending vibration of the upper torso with respect

to the lumbar spine.[18] Therefore, the natural frequency of the human body and that of heavy machinery like tractors are similar.[19] In a case control study, Kelsey *et al*.[20] described a significant association between herniated NP and truck driving with an odds ratio (OR) of 4.7.

The impact of vibration on disc cells has also been studied *in vitro*. In monolayer culture, Yamazaki *et al*.[21] cultured rabbit AF cells for six days. On the last day, cells were stimulated with a frequency of 6 Hz for 2, 4, 6 and 8 h. They found that gene expression for extracellular matrix molecules and matrix metalloproteinases was decreased. Alternatively, Kasra *et al.* cultured porcine IVD cells in alginate beads under dynamic hydrostatic loading at 1, 3, 5, 8 and 10 Hz with the same amplitude of 1 MPa.[22] They described a drop in DNA amount, impaired protein synthesis and increased degeneration at a frequency range of 3–8 Hz.

Although the response to specific stimuli such as nutrition and mechanical stress can be investigated in detail in isolated cells, removal from their native, highly specialized, extracellular environment doubtlessly affects the cell behavior.[23,24] Therefore, we aimed to investigate the impact of vibration conditions in an in vitro IVD organ culture system, which has not been demonstrated before. We have previously reported that whole IVDs with intact endplates can be maintained viable for up to three weeks when cultured under simulated physiological loading and sufficient nutrition conditions in our IVD organ culture system.[25] Moreover, limiting nutrition resulted in a decrease of viable cells within days and a new plateau at a lower cell density (50%) was established and remained stable for up to three weeks. This finding corroborates earlier studies and clearly supports the detrimental effects of impaired nutrition on the IVD.[26]

In this study, whole IVDs with EPs were exposed to high frequency (10Hz) loading in order to mimic deleterious vibration, which then was compared to “physiological” (0.2Hz) loading. Moreover, the influence of high frequency

load on IVDs cultured under "limited" nutrition conditions was also investigated to reproduce the experience of potentially detrimental loading in discs suffering from a compromised nutrition state, e.g. due to EP calcification. This allowed us to reveal potential interactive effects of excessive load and limited nutrition, which are both known etiologic factors in IVD diseases, in a whole organ culture. In contrast to physiological loading, which is likely to accelerate diffusion of nutrients into the disc, high frequency load, similar to a static loading situation, may even slow down this process (for review see Iatridis et al.[27]); since the time frame between loading periods is too short to allow significant fluid flow. We therefore hypothesized that combining high frequency loading with nutrient restriction may impair the disc cells in an additive or synergistic manner.

5.3 Material and Methods

The procedure for disc harvesting and preparation, as well as the bioreactor used in this study, were described previously.[28] For this study, discs were harvested from ten skeletally mature (2 – 5 yr-old) Swiss Alpine sheep (*Ovis aries*), which were already being euthanized for other experiments at our research institute. All pre-euthanization procedures were approved by the Animal Experimentation Commission of the Veterinarian Office of the Canton of Grison, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Five caudal discs were prepared from each sheep tail. To exclude disc level dependency of cell metabolism rates[29], the differently cultured as well as day 0 (d0) control discs were randomly distributed among the harvested caudal levels. The vertebrae were cut just proximal and distal to EPs with a histological band saw, discs were cleaned and loaded in bioreactors as previously described (Figure 11).[28] Discs were cultured for 7 days either under simulated-physiological (low) or high-frequency (high) loading, i.e. diurnal axial load (0.2/0.6 MPa, 8/16 h) with 2 x 4 h cyclic load during the 0.6 MPa-phase with simulated-physiological (0.2 Hz; ± 0.2 MPa) or higher-frequency

(10 Hz; ± 0.2 MPa) loading (Figure 18). Discs were cultured in DMEM containing 10% FCS and either limited (lim = 2 g/l) or sufficient (suf = 4.5 g/l) glucose concentration. Since the difference of osmolality between sufficient and limited media was only minor (6.5 mOsm/kg H_2O, measured in DMEM w/o FCS), osmolality of the media was not adjusted, avoiding possible interfering effects from substitutes.

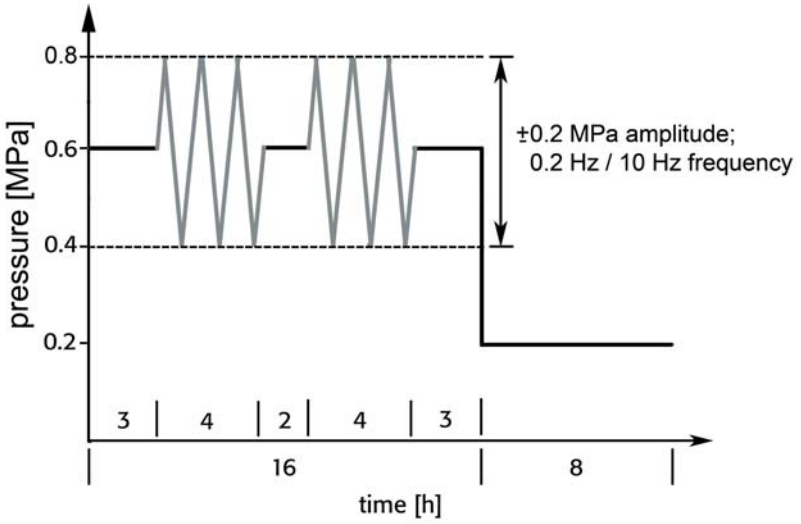

Figure 18: Loading scheme for culturing discs under simulated physiological or high frequency axial loading; One cycle requires 24 h: 16 h at 0.6 MPa mean stress, including 2 x 4 h sinusoidal cyclic compression at either 0.2 or 10 Hz with an amplitude of ±0.2 MPa; followed by 8 h rest at 0.2 MPa.

The following output parameters were measured in order to investigate whether limited nutrition and vibration, alone or in combination, have detrimental (catabolic) effects on the disc, resulting in cell death and ECM breakdown: i) cell viability;[25] ii) expression of anabolic and catabolic genes; iii) metabolic activity of the disc cells in terms of newly synthesized aggrecan; iv) glycosaminoglycan synthesis rate; v) collagen content and breakdown; vi) proteoglycan content; and vii) disc morphology.

After culture, EPs of discs were removed with a scalpel blade and the AF and NP were separated with a biopsy punch (diameter = 5 mm). Tissues were then cut into three equal parts: one third was used to assess cell viability; the second third was used to assess gene expression; and the last third was further split for

collagen breakdown, determination of aggrecan synthesis, glycosaminoglycan (GAG) synthesis rate, and proteoglycan content. Alternatively, three discs were cut in half with a band saw. One half was processed for histology and the other half was used to assess cell viability, gene expression or aggrecan synthesis.

Cell viability was determined with 5 μM calcein AM and 1 μM ethidium homodimer-1 (Molecular Probes, Leiden, The Netherlands). Disc tissue was incubated in serum free medium supplemented with the dyes under free swelling condition for 2 h. Stained samples were visualized on an inverted confocal laser scanning microscope (LSM510, Zeiss, Jena, Germany) and analyzed with ImageJ software (http://rsb.info.nih.gov/ij/) as described previously.[30]

To assess gene expression, disc tissue samples were flash frozen in liquid N_2, pulverized, and total RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), using a modified TRIspin[25] method. Reverse transcription was performed using TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA). Expression of selected anabolic and catabolic genes, i.e. aggrecan (ACAN), collagen I (COL1A1), collagen II (COL2A1), ADAMTS4, MMP7 and MMP13 was assessed by real-time RT-PCR (ABI GeneAmp 7500, Applied Biosystems), using 18S ribosomal RNA as the endogenous control[28,31] Unpublished sequences are as follows: MMP7: forward primer (5'-3') GTG GCC AAG GCC TTC AAA; reverse primer (5'-3') CTT CTT GCA AAG CCA ATC ATG A; probe (5'FAM-3'TAMRA) AGC GAA GCA ATC CCA CTG ACG TTT AAG A. Samples with undetectable expression of the target gene were assigned a C_t of 45 (maximum number of cycles), representing extremely low mRNA concentration.

Aggrecan synthesis in each tissue sample was assessed by measuring the amount of the chondroitin sulphate 846 (CS846) epitope of the proteoglycan aggrecan, as described previously.[25] The monoclonal antibody 846 recognizes a chondroitin sulfate epitope on the largest aggrecan molecules showing 100% aggregate ability with hyaluronan. The epitope content is barely detectable in

normal adult cartilage and discs, is increased in osteoarthritic cartilage and degenerated discs, and is highest in fetal and neonatal cartilage.[32,33] The proteoglycans were extracted in 1 ml of CHAPS buffer (4 M guanidinium chloride in 50 mM sodium acetate, 0.5% CHAPS (3-(3-cholamidopropyl) diethyl-ammonio-1 propanesulfonate), pH 5.8, with protease inhibitor cocktail, Sigma P8340) and dialyzed. Aliquots of 50 µl were lyophilized and dissolved in buffer supplied with the CS846 Epitope ELISA kit (IBEX, Montréal, Canada). Samples were treated and analyzed according to the manufacturer's instructions and concentrations of CS846 were normalized to the wet weight of the tissue.

GAG Synthesis Rate by ^{35}S Incorporation. Separate pieces of NP and AF were incubated "free-swelling" for 20 h in complete medium supplemented with 2.5 Ci/mL ^{35}S-sulfate ($Na_2{}^{35}SO_4$; Perkin Elmer Boston, MA, USA). The tissues were then digested in 1 ml 0.5 mg/ml proteinase-K solution. Five hundred µl of both, the radio-labeled digests and medium, were loaded onto a PD-10 desalting column (Amersham, Inc., cat. # 17-0851-01) and washed with an eluent solution (pH 7.5) comprising; 1M sodium chloride, 0.1 M anhydrous sodium sulfate, 0.05 M Tris/ hydrochloride and 0.5% triton X-100. Fractions of 500 µl were collected in 5 ml vials (Simports, Quebec, Canada), combined with 3.5 ml of scintillation fluid (OptiPhase HiSafeTM 3, PerkinElmer Inc., Regensburg, Switzerland) and the activity was counted using a liquid scintillation counter (Wallac 1414 Liquid Scintillation Counter, Perkin Elmer Inc.). The counts per minute were normalized to wet weight of the samples.

Other biochemical analyses.[34] The denatured collagen was solubilized with alpha-chymotrypsin and the remaining collagen chains were digested with proteinase K[33] Percent of denatured and total collagen were assessed by determination of the OH-proline content, in their respective digests.[35] Proteoglycan amount of proteinase K digested tissue and of culture media was measured with the DMMB dye assay.[33-35] Total water retained in the tissue was also determined.

For Histology, specimens were fixed in 4% buffered formaldehyde, decalcified in EDTA, and embedded in paraffin. Sagittal sections (6 µm thick) were stained with Safranin-O/Fast green.

For statistical analyses, gene expression and biochemical data were normalized to the values of corresponding d0 control discs. For statistical analyses univariate GLM with subsequent pairwise *post hoc* testing was performed (Fisher's LSD and Games-Howell). For all statistical analyzes a p-value < 0.05 was considered significant.

5.4 Results

Cell viability in fresh discs was 86.02 % (84.36-89.89) in annulus fibrosus (AF) and 92.24% (90.75-95.97) in nucleus pulposus (NP) [values are median (IQR)]. For discs cultured in sufficient media under simulated-physiological (suf/low) conditions, the viability was maintained [AF = 85.35 % (81.98-94.72), NP = 89.77 % (84.46-92.74)]. Whereas culturing under limited condition or high frequency load resulted in decreased cell viability [suf/high: AF = 61.87 % (46.52-79.24), NP = 55.04 % (48.39-66.16); lim/low: AF = 65.78 % (49.74-73.97), NP = 75.84 % (70.90-82.15)]. In the NP, high frequency load had a greater impact on the cell death than limiting nutrition. Combined culture under limited glucose condition and high frequency load had an additive increase in cell death [lim/high: AF = 39.63 % (33.67-49.53), NP = 57.61 % (42.91-62.80; Figure 19)].

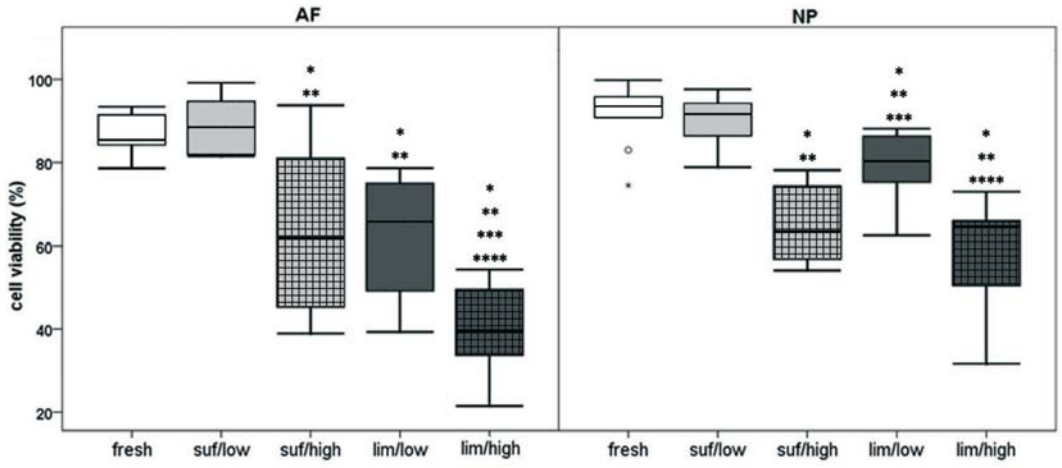

Figure 19: Cell viability of annulus fibrosus (AF) and nucleus pulposus (NP) after seven days culture. In both tissues, cell viability could be maintained when cultured under suf/low conditions whereas it dropped significantly in the stressed discs. *notes = significant differences ($p < 0.02$); * = compared to fresh; ** = compared to suf/low; *** = compared to suf/high; **** = compared to lim/low.

Gene expression analysis revealed an increase in MMP13 mRNA that was significant for the NP, in discs cultured under lim/high conditions (NP: $p = .046$; AF: $p = .064$ vs. suf/low). For the other genes, no differences were obtained. Notably, in the NP, MMP7 seemed to be slightly up-regulated when cultured under lim/high conditions; however, this did not reach significance (Figure 20).

Aggrecan synthesis was not significantly different between discs cultured in different conditions, neither for AF nor for NP tissue (Figure 21). GAG Synthesis Rate by ^{35}S Incorporation did not reveal any changes between groups. Neither nutrition nor loading frequency had a noticeable effect on GAG synthesis rates after seven-day culture, although for the AF, ^{35}S incorporation tended to be slightly increased when cultured under limited nutrition (data not shown). Similarly, denatured collagen, water content, GAG content in tissue or GAG released in media did not reveal any significant differences between culture conditions.

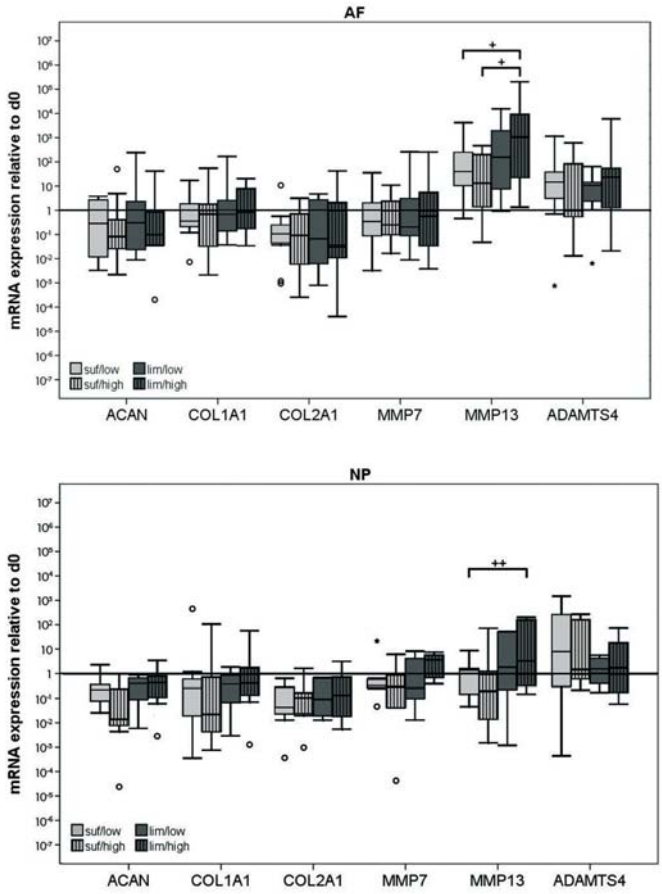

Figure 20: Relative gene expression of three anabolic and three catabolic genes after seven days culture. A = annulus fibrosus (AF) B = nucleus pulposus (NP). RT-PCR data were normalized to house-keeping gene (18S rRNA) and are presented relative to d0 fresh control. Compared to suf/low-cultured discs MMP13 was more highly expressed in the stressed discs under lim/high conditions (+ is $0.05<p<0.065$; ++ is $p<0.05$).

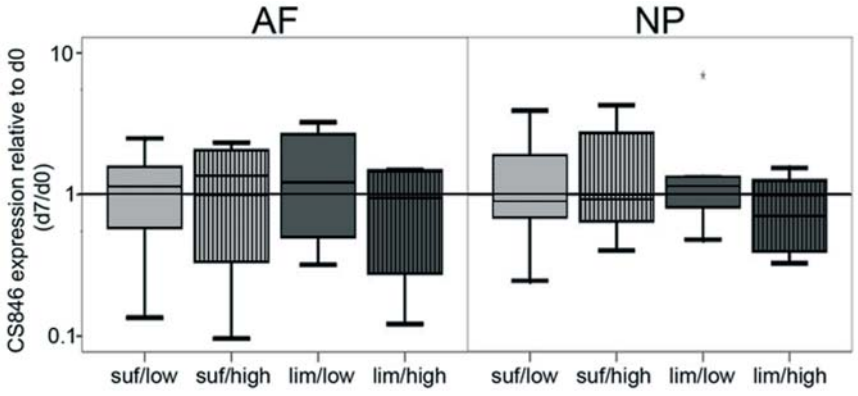

Figure 21: Boxplots of CS846 expression relative to d0. Neither in annulus fibrosus (AF) nor in nucleus pulposus (NP) significant differences were detected between culture conditions or compared to fresh discs.

Morphology of the discs could be maintained during the culture. Analyses of the Safranin-O/Fast green stain revealed neither rupture of the AF nor cracks in the EP after any culture condition. However when cultured under high frequency load the AF bulged slightly outward compared to fresh discs or discs cultured under lower simulated-physiological loading frequencies (Fig. 6).

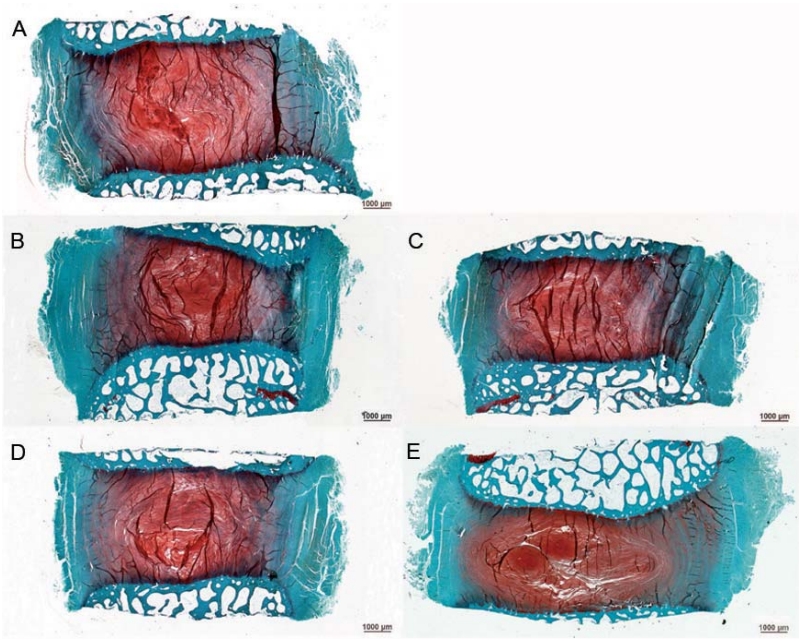

Figure 22: Sections of Safarin-O/fast green stains of discs before and after 7 days of culture. A = d0; B = suf/low; C = suf/high; D = lim/low; E = lim/high.

5.5 Discussion

This study is based on previous work where we cultured caudal ovine discs in an organ culture system with simulated-physiological loading and media containing sufficient or limited concentration of glucose. There, cell viability could be maintained up to three weeks if adequate glucose was provided. Although decreasing glucose concentration did cause substantial reduction in living cells, it was not sufficient to provoke other indications for disc degeneration at the gene expression and protein levels during 7 and 21 days of culture. In the

present study, in addition to limited nutrition, the impact of high frequency (10 Hz) load was investigated. Exposure to a frequency of ~10Hz can be experienced when flying in a helicopter and is similar to the natural vibration frequency in a human body.[18] In a questionnaire survey Bongers et al.[36] investigated the prevalence of LBP of helicopter pilots and found that it correlated with total flight time (OR 13.4). In a laboratory simulation, Pope *et al.* reproduced the environment of a helicopter and measured a loss of comfort of the volunteers already after a 2 h exposure.[18] We therefore hypothesized that 7-day culture under high frequency load would induce metabolic changes in our system. Disc degeneration is caused by multiple factors. These can be genetic or epigenetic such as age-related or physiological or linked to living or occupational circumstances. Therefore, we specifically aimed to investigate the single and combined effect of high frequency load as an environmental factor and limited nutrition as an intrinsic factor, resulting for example from endplate calcification. In particular, vibration-like loading may reduce the diffusion of nutrients and at the same time enhance the accumulation of acidic metabolites within the disc. If nutrient supply is already diminished, this may lead to accelerated cell death. Interestingly, high frequency compression had a greater impact on the NP cells than limiting nutrition, while the AF cells were affected to a similar extent by both stimuli. This outcome essentially results from a less pronounced effect of nutrition deficiency on the NP than AF cells. NP cells are physiologically exposed to harsh conditions and may therefore be more resistant to nutritional challenges than AF cells.[37,38] Detrimental load, however, equally affected both AF and NP cells. The combined parameters (lim/high) increased the cell death in an additive manner, resulting in lowest viability in the AF of lim/high-cultured discs. These data, extrapolated to the human situation, could mean that subjects with IVD nutrition problems, which are additionally exposed to high frequency vibration sources (~10 Hz) might be more likely to suffer from disc degeneration with respect to increased cell death. Although our data may suggest that impaired nutrition and excess load may separately contribute to changes in the IVD cell viability, it is

important to note that these parameters cannot be regarded as completely independent. It has been shown in animal models that vibration itself may lead to compromised nutrition.[39,40] Hence, when combined with limited glucose supply, vibration may affect the disc cells in a synergistic manner. Analysis of additional parameters addressing the nutrition state of the disc cells will be required to elucidate the damaging mechanisms of vibration stress more in depth.

Although cell viability assays can evaluate the survival rate of cells, they may not assess their metabolic activity. The GAG synthesis rates we obtained for fresh discs and discs cultured under sufficient nutrition and low frequency are in line with those reported by other investigators, who also documented higher values in the NP compared to the AF.[26,29,37,41] In addition, comparing the different culture conditions, no changes were obtained for the NP after seven days. According to the heightened nutrient sensitivity of the AF with respect to cell viability, the slight increase of ^{35}S incorporation in the limited cultured discs could indicate a first metabolic response of the challenged discs. Since our experimental set-up did not allow us to measure the sulfate incorporation during culture, the ^{35}S incorporation was determined while the tissue was in free-swelling conditions, which might affect synthesis rates. Thus, our comparisons were based only on relative values (between groups). Moreover, we additionally measured the amount of newly synthesized aggrecan by CS846 quantification. *In vitro* studies have shown a direct correlation of the content of this epitope in cartilage to aggrecan synthesis rate measured by ^{35}S incorporation into GAG.[42,43] The half-life of CS846 has been estimated to be 15-20 h.[44] Hence, it may be considered to be representative of a newly synthesized subpopulation of aggrecan molecules during culture time.[33] Still, corresponding to the ^{35}S incorporation, no significant differences could be obtained between culture conditions (Figure 21).

With the up-regulation of MMP13, the discs responded directly at the gene expression level to the additive culture (lim/high). Remarkably, other catabolic genes, i.e. ADAMTS4 and MMP7 were not up-regulated after the 7-day culture. During IVD degeneration, the expression of several matrix-metalloproteinases

increases, including MMP1, -3, -7, -9 and -13, all known to degrade many of the main matrix components.[45-47] It has been shown that of these MMPs, MMP7 and -13 (whose predominant targets are collagen type II and aggrecan) are most highly expressed within degenerated discs, particularly in the NP.[45,46] However, since MMPs are secreted in latent form (pro-MMPs) and their activation is regulated by specific TIMPs (tissue inhibitors of metalloproteinases), which inhibit certain particular MMPs[48] the pro- and activated forms are indistinguishable from each other by means of relative gene expression analysis. Le Maitre *et al*. reported that MMP7 is more resistant to TIMP-1 and -2 deactivation and, therefore, might have a key function in terms of disc matrix remodeling.[46] As a result, MMP7 may be activated in the stressed disc at the post-transcriptional or even post-translational level. Hence, inactive pro-MMP7 present in the non-degenerated disc may be converted to the active isoform in the stressed discs, which cannot be detected at the mRNA expression level. In contrast, MMP13 is hardly expressed in non-degenerated discs but increases with degeneration,[45] which is in line with our observed increase in MMP13 expression and points to an early sign for a response of the stressed discs.

Due to the clear increase of cell-death after the "stressing" culture, one could assume a greater response in gene expression and extracellular matrix turnover by the disc cells. The work of Korecki et al[49] supports our findings in this respect. They cultured young and mature bovine disc cells in 3D alginate, and exposed them to frequency load for seven days (0.1 Hz, 1 Hz and 3 Hz compared to free swelling). Exposure of mature bovine disc cells to frequency load did not result in changes, neither in DNA nor in GAG content. Responses in gene expression were only minor for AF cells, while for NP cells, no effect of loading was observed. Similar findings were observed by Wuertz *et al*. in an *in vivo* rat tail model. Even after eight weeks with daily loading for eight hours at 1 MPa and 1 Hz frequency, only a mild degenerative shift was observed when compared to unloaded discs.[50]

Although changes were detected at the cell viability and gene expression level, our harsh culture conditions of limiting nutrition combined with a frequency of 10 Hz are apparently not sufficient to induce disc degeneration at the protein level during a short-term whole organ culture. With approximately $2x10^6$ cells/g wet weight in fresh discs, cell density is relatively low, limiting cell-to-cell signaling; consequently, the single cell cannot sense that the surrounding cell is stressed. Moreover, potential alterations in the ECM composition or turnover might be below the detection limit of the applied biochemical assays. It has to be considered that relatively few cells are responsible for a comparably immense amount of ECM. Assuming the cell viability decreases from ~100% to ~50% in a bio-mechanically stressed disc, the differences in matrix production would be hardly detectable, even if the remaining cells extensively increased their metabolic activity.

The inter-animal variation was minimized since only study animals from the same breeder were used. However, these sheep were between two and five years of age, which may imply age related differences, with a relatively small sample size. Nevertheless, it is conceivable that if the cells were given more time to respond to the changing conditions, e.g. by increasing the culture time, one could expect a stronger response also on ECM level. Therefore, further experiments will investigate effects of extreme high (10Hz) frequency loading and varying nutrition in a mid-term culture (i.e. three week culture).

Current treatment strategies for discogenic pain are generally limited to spinal fusion or mechanic implants and little is known about biological regeneration strategies, e.g. cell or growth factor therapy. *In vitro* models for investigating potential preventive or regenerative therapies are rare even though it is desirable to test the applicability of new treatment strategies in an environment simulating *in vivo* conditions. In this context, our in vitro whole organ IVD culture system may be considered as a valuable tool not only for investigating potential mechanisms leading to the onset of IVD degeneration (excessive loading and limited nutrition), but also for evaluating new regenerative strategies, e.g. by

monitoring the fate (survival and differentiation) of injected mesenchymal stem cells or the impact of growth factor therapy.

5.6 Acknowledgement

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Homing of Mesenchymal Stem Cells in Stressed Intervertebral Discs

Svenja Illien-Jünger, MSc[a], Sibylle Grad, PhD[a], Daisuke Sakai PhD[b], Joji Mochida PhD[b] Mauro Alini, PhD[a]

[a] *AO Research Institute, Davos, Switzerland*
[b] *Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan;*

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6.1 Summary

Study design. Bovine caudal intervertebral discs (IVD) were co-cultured with human bone marrow derived mesenchymal stem cells (BMSC) and homing of BMSC into the disc was investigated.

Objective. To investigate in a whole organ culture whether metabolically and mechanically challenged discs release factors that may attract human BMSCs.

Summary of background data. It has been shown that BMSCs survive within the IVD and that cells from injured tissues release cytokines and mediators that are involved in the healing process. One important function of these factors is the recruitment of progenitor cells, which then contribute to tissue repair.

Methods. IVDs were cultured under "simulated-physiological" or "degenerating" nutrition and loading conditions. For the latter, one disc/group was stabbed with a 22g needle in the IVD center to create a defect. Culture media was collected for chemotaxis analyzes. PKH26 labeled human BMSCs were added to the bioreactor chamber hosting the discs and were co-cultured with the IVDs for an additional 8 days. After culture, engraftment of BMSCs into the discs was microscopically analyzed; chemoattraction of culture media was assayed with a chemotaxis Boyden chamber.

Results. Compared to IVDs cultured under "simulated-physiological" conditions, the number of BMSCs migrating towards the center of the disc significantly increased three to four times in "degenerating" IVDs. Amounts of recruited BMSCs were highest in most stressed discs. With the double amount of migrating cells compared to the basal migration, media of "degenerating" discs also demonstrated chemo-attractive activity towards hBMSCs

Conclusion. In this study we showed in an *ex vivo* organ culture that "degenerative" conditions may induce the release of factors that promote the recruitment of BMSCs. Since regenerative effects of attracted BMSCs can be

assumed, this finding has implications for therapeutic delivery of BMSCs and endogenous progenitor cell activation.

6.2 Introduction

Adult mesenchymal stem cells (MSCs) are increasingly recognized as a promising source of stem cells for tissue repair and regeneration therapies. MSCs can be isolated from various tissues including bone marrow, adipose tissue, skeletal muscle, periostum, and synovium, with bone marrow and adipose being the most studied sources. In fact, it has been suggested that cells with MSC-like properties can be identified in virtually all postnatal tissues and organs. Major characteristics of MSCs are their capacity for rapid proliferation, while retaining their multilineage potential, and for differentiation into a number of phenotypes including bone, cartilage, muscle, ligament, tendon, adipose, and stromal tissues.[1] Besides these mesenchymal cell types, it has also been documented that MSCs can be differentiated into neural cells *in vitro* and are able to express a cardio-myogenic phenotype.[2,3] Furthermore, both *in vitro* and *in vivo* studies have indicated the potential of MSCs to differentiate towards the phenotype expressed by nucleus pulposus cells of the intervertebral disc (IVD).[4-7] In addition, MSCs are documented to have immunosuppressive properties and are regarded as non-immunogenic, rendering them particularly attractive for tissue engineering, cell and gene therapies.[8,9]

While MSCs have been extensively characterized in *in vitro* studies, there is less information available about their *in vivo* behaviour. However, both site-directed and systemic administrations of these cell types have indicated their engraftment into different tissues, especially after injury or disease states. It has been shown that systemic delivery of MSCs results in their specific migration to a site of injury. The ability of MSCs to locate areas of tissue damage has been demonstrated in bone defects,[10] myocardial infarction,[11,12] ischemic cerebral injury,[13,14] nephropathy,[15] pulmonary fibrosis, and wound healing.[16] Migrating

MSCs may thus represent a source of pluripotent cells that are available for repair of diseased or injured tissues and organs. The mechanisms that guide the recruitment of these cells are still not clarified yet. However it is suggested that trophic factors are involved. Several studies have reported the functional expression of various chemokine receptors on MSCs,[17-19] and inflammatory cytokines have been shown to trigger chemotactic migration of MSCs through extracellular matrix structures.[20]

These findings might suggest that homing of MSCs to damaged or diseased areas within the IVD may also occur. It has been shown that the cells of the disc initially react to certain injurious stimuli by enhanced proliferation and metabolic activity.[6,21] Up-regulation of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) has been observed in damaged discs and after unphysiologically high mechanical loading periods.[22,23] Moreover, increased expression of proinflammatory mediators has been demonstrated in disc degeneration and particularly upon repeated injury of the intervertebral disc.[22,24] While these reactions may lead to further damage of the disc, they may also stimulate endogenous repair mechanisms, including recruitment of stem or progenitor cells. There is increasing evidence that implantation of MSCs into damaged discs may regenerate the tissue by both activating endogenous disc cells and differentiating towards IVD-like cells.[25,26]

We therefore hypothesized that bone marrow derived human MSCs will be attracted more effectively by IVDs maintained in a whole organ culture system under "degenerating" conditions as compared to simulated-physiological conditions. In previous studies we could show that both limiting nutrition as well as applying high frequency mechanical load reduced the cell survival rate within cultured discs, mimicking the condition of a (early) degenerating disc.[27,28] The present work hence investigates the migration of human MSCs from the environment towards the center of a mechanically and nutritionally challenged "degenerating" disc. Since it is suggested that chemotactic factors released by the disc cells may play a role, the chemotactic activity of conditioned media from the

disc cultured under "degenerating" conditions towards human MSCs was also assessed

6.3 Materials and Methods

6.3.1 Cultures

Pre-culture of intervertebral discs: For this study, intervertebral discs were harvested from young (5-8 month old) bovine tails that were obtained from the local abattoir within 2 hours of death. A total of 2-4 discs were excised from each tail. For dissection of the discs, surrounding tissue was removed and discs were harvested by cutting along the proximal and distal cartilage endplate (CEP) boundaries with a scalpel. Excised discs were kept moist in gauze saturated with phosphate buffered saline (PBS) until further processing. To exclude the possibility of disc level dependent cell metabolism rates[29], the cultured discs were distributed among the harvested caudal levels. IVD were rinsed with PBS. All discs were rinsed for ten minutes in a solution of PBS containing 10% penicillin-streptomycin (P/S; 1,000 U/ml penicillin, 1,000 µg/ml streptomycin), followed by another rinse in PBS before placing in bioreactor chambers as previously described.[28] The harvested discs had average diameters ranging from 10 to 15 mm. IVDs were cultured for 7 days in pre-culture (as previously described; Figure 23).[27] In short: disc were placed under axial load (0.2/0.6 MPa, 8/16 h) with 2 x 4 h cyclic load during the 0.6 MPa-phase with either simulated-physiological (0.2 Hz; ± 0.2 MPa) or higher-frequency (10 Hz; ± 0.2 MPa) loading. Additionally, one disc/experimental condition was stabbed with a 22g needle in the center of the IVD to create a defect and cultured as described below. (Figure 23). The different pre-cultures were preformed to determine the appropriate combination of detrimental factors to induce different stages of degenerating discs within a short culture time by simultaneously creating an environment that still might attract BMSCs to migrate into.

IVDs were cultured in 2.5 ml DMEM containing 1% ITS and 0.05% Primocin, with either limited (lim = 2 g/l) or sufficient (suf = 4.5 g/l) glucose concentration. Since the difference in osmolality between sufficient and limited media was only minor (6.5 mOsm/kg H_2O, measured in DMEM w/o ITS), osmolality of the media was not adjusted, avoiding possible interfering effects from substitutes. The first media change was performed at day one, thereafter medium was renewed and collected for chemotaxis analyzes every other day.

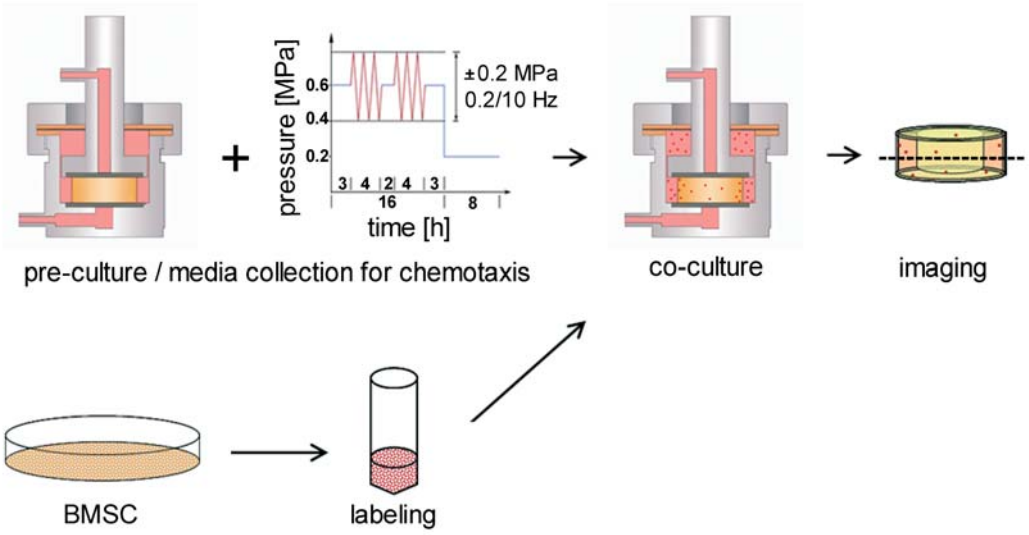


Figure 23: Culture scheme of bovine IVD co-cultured with hBMSC: IVDs are pre-cultured for 7 days and culture media is collected for chemotaxis analyses; then, discs are co-cultured with labeled BMSCs for 8 days and BMSC engraftment is imaged microscopically.

Labeling and administration of hBMSCs: Human bone marrow was obtained from the University Hospital of Bern after approval by the local ethical commission and written consent of the patients (n = 3). Bone marrow derived mesenchymal stem cells (BMSC) were isolated by Ficoll® gradient centrifugation as described before.[30] Early passages (p1-3) of BMSCs were trypsinized and washed in DMEM containing 10% fetal bovine serum (FBS; Gibco) to neutralize the trypsin/EDTA solution. Washing was repeated with DMEM without FBS.

Then the cells were stained with the fluorescent membrane dye PKH26 (PKH26 Fluorescent Cell Linker Kit; Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions with some modifications. In brief, $8x10^6$ hBMSCs were transferred in a 15 ml tube and re-suspended in 500 µl diluentC (PKH26 Fluorescent Cell Linker Kit) and immediately mixed with an equal volume of 0.6% PKH26 stock solution in diluentC. The suspension was incubated on a wobbling device for 10 minutes at room temperature. To stop the staining reaction, an equal volume (1 ml) of FBS was added to the cells. After one minute, DMEM was added, hBMSCs were pelleted again and transferred to a fresh 15 ml tube and washing was repeated twice with DMEM without FBS. Finally, cells were re-suspended in 240 µl DMEM. Viability was over 90% as determined by trypan blue exclusion.

In a pilot study, the survival of BMSCs in the above mentioned culture environments was tested. Cell viability of BMSCs dropped already significantly when cultured under physiological load and decreased even further when frequency load was applied (data not shown).

Therefore, the co-culture of IVDs and BMSCs was preformed under minimal static load. To provide the same initial situation for co-culture, all groups were cultured with sufficient nutrition supply. After 7 days pre-culture, medium was removed from the IVD culture chamber and 30 µl ($1x10^6$ cells) hBMSC suspension was added onto the upper surface of each disc. After 20 minutes, 2.5 ml DMEM was added into the bioreactor chamber, the system was closed again and placed into the loading device. Following a 30 minute incubation time at 0.5 N (to avoid swelling of the IVD), discs were cultured under 0.1 MPa static load for 8 days. Fresh BMSCs ($1x10^6$ cells/disc) were added after 2 and 4 days and media change was performed every other day. BMSCs were also added to freshly harvested IVD according to the same procedure, to investigate if already 7 days pre-culture under physiological conditions affects BMSC engraftment (Figure 23).

To analyze whether a potentially increased engraftment into stressed discs would be only due to the mechanical impact of high frequency and needle puncture, additional discs were frozen at -20°C for 2 weeks to induce cell death. The dead discs were punched with a 22g needle and cultured under sufficient nutrition and high frequency load for 7 days. Then cells were added as described above. After culture, IVDs were rinsed in PBS, transferred in 10% Trypsin/EDTA and incubated on a wobbling device for 5 minutes at room temperature to remove BMSCs attached to the disc surface. Fixation of the discs was performed in 4% buffered Formalin for 3 days at 4°C. After washing 3 times in PBS, IVDs were cut transversely and washed again twice.

6.3.2 Analyses

Disc diameter and height was measured twice: i.e. before culture, directly after disc dissection before further cleaning of the tissues; and after 15 day culture, directly after taking the discs out of the bioreactors. The dimensions were determined with a sliding caliper (n = 4).

Engraftment of BMSCs into the discs was analyzed microscopically. The entire disc area was visualized on a motorized microscope (Axiovert200m, Zeiss, Jena, Germany) at 2.5 times magnification (Plan Neofluar objective, 2.5x/0.075; fluoresce filter: Zeiss #15; Axiovision 4.5). The final image (MosaiX, Zeiss, Jena, Germany) was composed of several single screens (1388x1040 pixel; resolution 2.420048 µm/pixel) and depended on the disc size. The BMSC engraftment was analyzed interactively by determining the area of interest and marking the cells (suf/low+p: n = 5; n = 4 for the other conditions; Figure 24).

Sagittal sections of IVDs were also analyzed in order to get an impression of the migration paths of BMSCs through NP and AF (n = 4 each for suf/low and suf/low+p) and imaged as described above.

Viable cells were identified with 5 µM calcein AM (Molecular Probes, Leiden, The Netherlands). Disc tissue was incubated in serum free medium supplemented with the dye under free swelling condition for 2 h (n = 4 each for

suf/low and suf/low+p). Stained samples were visualized on an inverted confocal laser scanning microscope (LSM510, Zeiss, Jena, Germany)

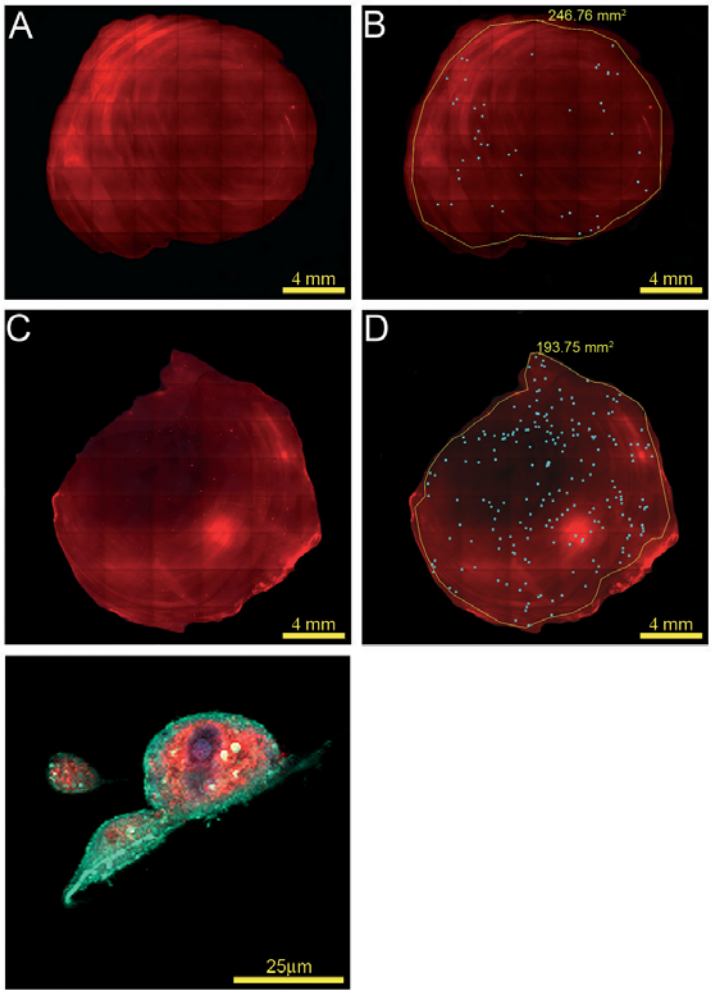


Figure 24: A Engraftment of BMSCs into the disc; Imaged bovine IVD, pre-cultured suf/low (A+B) or lim/high + puncture (C+D) before (A+C) and after (B+D) analyzes; yellow ring marks the region of interest; blue dots mark the counted cells. For analyses cells/100mm^2 are calculated B: Morphology of a labeled BMSCs in the 3D environment of a fixed disc; red = membrane stain (PKH26); green = actin (Alexa Fluor® 488 Phalloidin); blue = nucleus (TO-PRO®3 iodide)

Morphology of migrated BMSCs inside the disc was assessed by fluorescent labeling. Because of the sensitivity of PKH26 for detergents, the intracellular structure was made accessible as follows: after fixation, the transverse disc sections were transferred into PBS containing 30% sucrose overnight at 4 °C. Discs were treated for staining by successive freeze/thaw cycles

in liquid nitrogen.[31] To make the actin skeleton visible, the sections were stained 1:100 with Alexa Fluor® 488 Phalloidin (Invitrogen A12379) in PBS for one hour at room temperature. The nuclei were labelled 1:1000 with TO-PRO®3 iodide (Invitrogen T3605) in PBS for 20 minutes. Stained samples were visualized on an inverted confocal laser scanning microscope (LSM510, Zeiss, Jena, Germany).

Chemo-attraction of the conditioned media of discs cultured under different loading schemes was analyzed with a 48-well chemotaxis Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) as previously described.[32] For the chemo attraction experiments only conditions of the same glucose concentration could be compared. Therefore, conditioned media of simulated-physiological ("normal") and stabbed-high-frequency ("stressed") cultures were compared. The migration assays were performed in triplicate. Concentration gradients of media from "normal" and "stressed" cultures raised in opposing trends from 0%, 25%, 75%, and 100%. Media was warmed to 37°C and degassed by vortex. Increasing concentrations of "stressed" media were arranged in columns and added to the bottom chamber wells (26 µl/well). Media of the same concentration gradients containing unconditioned hBMSCs were arranged in rows and filled in the upper wells (50 µl). BMSCs were seeded in a final concentration of $2x10^4$ cells/well. The chemotaxis chamber was incubated at 37°C, 5% CO_2 and 90% humidity for 6-7 hours. After culture, the non-migrated cells were wiped from the membrane by drawing the filter up over a wiper blade. The migrated cells at the lower surface were fixed in 100% methanol and stained with 0.5% Toluidin blue. Migration was quantified by counting the number of migrated BMSCs. All culture wells were imaged with a motorized microscope (Axiovert200m, Zeiss, Jena, Germany) at 5 times magnification (Plan Neofluar objective, 5x/0.15; Axiovision 4.5). The composed image (MosaiX, Zeiss, Jena, Germany) consisted of 12 single images (1388x1040 pixel) and had a resolution of 1.2564 µm. The amount of migrated BMSCs was analyzed interactively by marking the cells. Each condition was performed in triplicate per assay, and the assays were repeated 3 times. Results are expressed as the mean number (±SEM)

of cells migrating towards "stressed" medium relative to the cells migrating towards "normal" medium (basal migration without "stressed" stimulus).

For statistical analyses, the number of migrated BMSC per area was normalized to the values of corresponding suf/low cultured discs from the same animal. Chemotactic activity was normalized to the corresponding basal migration within two identical media. For statistical analyses, the experiments were blocked per animal and univariate GLM with subsequent pairwise *post hoc* testing were performed (Fisher's LSD). For all statistical analyzes a p-value < 0.05 was considered significant.

6.4 Results

BMSCs homing in discs did slightly increase when pre-cultured under sufficient nutrition (suf) and simulated-physiological loading (low) compared to fresh discs; however, this was not significant (n = 4). Moreover, compared to suf/low, culturing under high frequency loading (high) and combining high frequency load with limited nutrition (lim) resulted in significantly increased engraftment of BMSC into the disc (lim/high: p=0.046; n = 4). This effect was more obvious when puncturing (p) the disc (suf/high+p p=0.014; n = 5) and was even higher when combining all stressing conditions (lim/high+p: p=0.006; n = 4; Figure 25; A+B). A basal BMSC engraftment was also observed into fresh and dead discs (n = 4). In sagittal sections of NP and AF of 4 sulf/low and suf/low+p cultured discs, migration through the NP as well as through outer AF was observed (Figure 26).

IVD dimensions slightly changed during culture (n = 4). Before culture the disc diameter was in average 12.7 mm ±0.69 and increased about 3.6 mm ±0.38 (suf/low) respectively 2.7 mm ±0.22 (suf/high+p). Whereas with an average height of 3.7 mm ±0.72 before culture and a decrease of around 1.19 mm ±0.38

(suf/low) respectively 0.77 mm ±0.67 (suf/high+p) the discs complanate after culture.

A

exp	fresh	dead	suf/low	suf/high	lim/high	suf/high+p	lim/high+p
1	ND	ND	34.69	33.01	53.76	ND	73.96
2	ND	ND	29.05	85.14	74.60	ND	79.73
3	ND	ND	25.51	53.54	108.46	ND	125.68
4	ND	ND	66.06	133.29	126.17	ND	237.52
5	89.67	91.06	127.86	ND	ND	272.04	ND
6	32.42	47.14	90.01	ND	ND	516.33	ND
7	59.19	39.52	285.71	ND	ND	ND	ND
8	69.72	74.69	238.28	ND	ND	300.73	ND
9	ND	ND	20.57	ND	ND	38.68	ND
10	ND	ND	20.80	ND	ND	71.37	ND

B

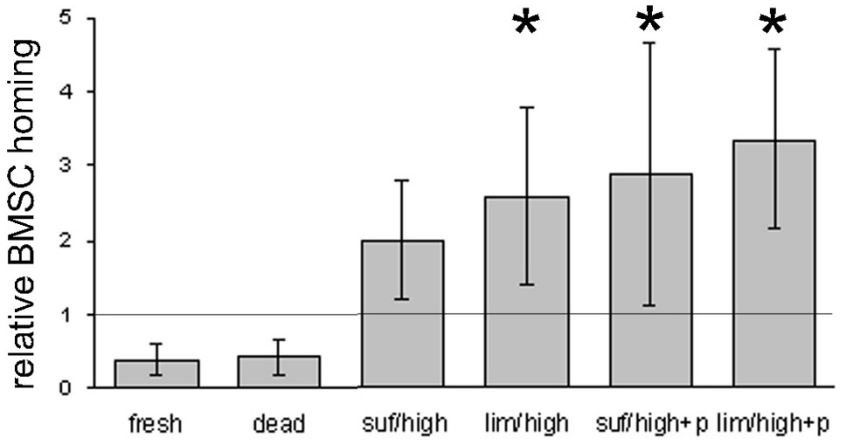


Figure 25: Evaluation of BMSC homing into the IVD; A: total BMSC engraftment of the different experimental groups (BMSC are presented in cells/100 mm^2), ND = not determined; a total of 10 experiments was preformed, each condition relative to suf/low B: Diagram of fold increase/decrease of BMSC homing relative to suf/low pre-culture (fresh = 0.39 ± 0.22; dead = 0.42 ±0.25; suf/high = 2.00 ±0.81 lim/high = 2.57 ±1.20; suf/high+p = 2.87 ±1.78; lim/high+p = 3.35 ±1.21; values are mean ± standard deviation); fresh = IVDs co-cultured with BMSCs under static load without pre-culture, dead = IVDs frozen at -20°C for 2 weeks, pre-cultured under suf/high+p; suf = sufficient nutrition; lim = limited nutrition; high = high frequency (10 Hz); low = low frequency (0.2 Hz); P = disc punched with 22g needle (* is $p<0.05$).

Viable BMSCs as well as NP and AF cells were observed within the entire disc of suf/low and suf/high+p cultured discs after 15 days culture (Figure 27).

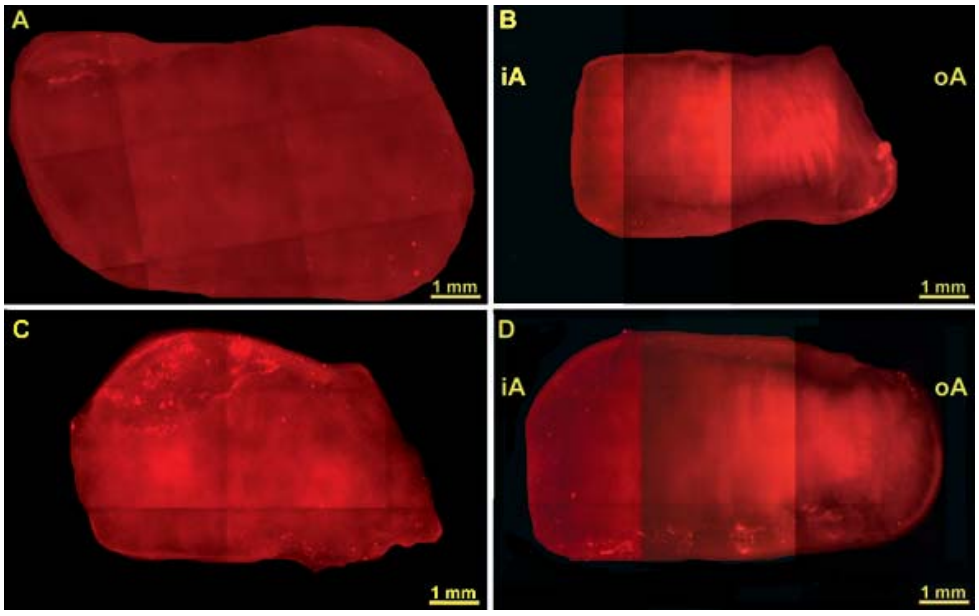


Figure 26: Representative images of sagittal sections through NP (A,C) and AF (B,D) after co-culture of suf/high (A,B) and suf/high+p (C,D) cultured discs with BMSCs. BMSC migration occurred through the NP directly and through the outer AF; iA = inner annulus fibrosus; oA = outer annulus fibrosus

Morphology of the labeled BMSCs in the 3D environment of the fixed disc appeared to be slightly roundish, the cytoskeleton and the nucleus were clearly visible (Figure 24).

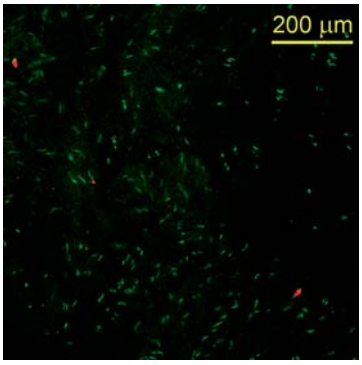
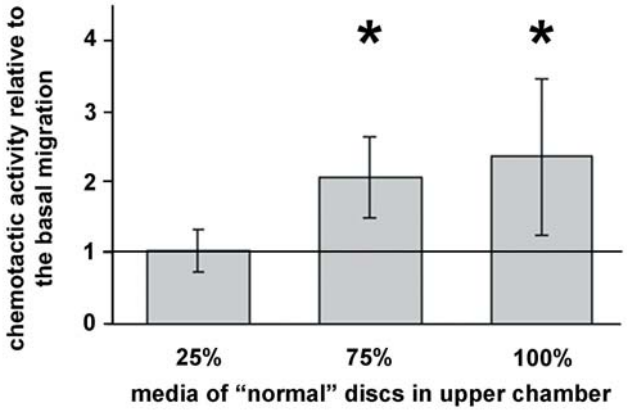


Figure 27: Representative image of viable cells after culture in the NP of a suf/high+p cultured disc co-cultured with BMSCs; green = viable NP cell, orange = viable BMSC

A

exp.	media of "normal"cultures in upper chamber 0%	25%	75%	100%
1	38.7	34.3	72.5	178.5
2	36.7	39.5	74.0	57.7
3	127.3	195.3	349.0	324.7
4	13.0	15.5	38.0	39.5
5	27.5	20.0	51.0	67.0
6	10.7	9.0	15.7	21.0

B



C

	↓ 100%	↓ 75%	↓ 25%	↓ 0%
→ 0 %	34.0	44.0	36.3	38.7
→ 25 %	39.0	62.0	55.5	34.3
→ 75 %	63.0	43.7	40.7	72.5
→ 100%	67.3	84.7	171.0	178.5

Figure 28: Analyzes of the chemotactic impact of conditioned culture media on BMSCs; **A**: total BMSC migration through the membrane (cells/8mm^2) **B**: Evaluation of the BMSC migration relative to the basal migration in "stressed" cultured media; results are presented as the percentage of "normal" culture media in the upper chamber with 100% "stressed" culture media in the lower chamber; 25% = 1.0 ±0.3; 75% = 2.1 ±0.6; 100% = 2.3 ±1.1 (values are mean ±SD; * is $p<0.05$) **C**: Representative example of checkerboard analyses for chemotactic activity in culture media; columns = percentage of conditioned medium from normal discs in lower chamber; rows = percentage of conditioned medium from normal discs in upper chamber, blue column = values taken into account for determining the chemotactic activity.

For chemotactic activity, only media from the same glucose concentration could be compared. Therefore, the chemo-attractive activity of conditioned media from sufficient cultures were taken into account (n=6). Chemo-attractive activity was observed for media collected from "stressed" discs (suf/high+p). The chemotactic adduction of "stressed" media for BMSCs in 100% and 75% "normal" media was ~2 times higher compared to the basal migration (100%: p=0.001; 75%: p=0.005) whereas no significant differences between 0% and 25% of "normal" media was observed (p=0.906; Figure 28).

6.5 Discussion

Next to the capability to differentiate into various cell types, i*n vivo* transplantation experiments with BMSCs indicated that those cells have the ability to migrate towards sites of injury and aid wound healing and tissue repair;[16] this progenitor mobilization is mainly promoted by cytokines/chemokines, adhesion molecules as well as proteolytic enzymes.[33] Based on this, we hypothesized that degenerating discs release factors that attract BMSC and induce their homing into the stressed tissue. The "ex vivo" induction of degeneration in discs is based on previous work, describing the development of an organ culturing model. Ovine caudal discs were cultured at simulated-physiological load and cell viability could be maintained up to three weeks, whereas reduction of glucose did cause a drop in cell viability of 50%.[28] The influence of nutrition demission was also shown *in vitro.* Horner *et al*. cultured bovine nucleus pulposus (NP) cells in an agarose gel diffusion chamber. After comparing different cell densities, oxygen and glucose concentrations they could show that a fall in nutrient supply reduced the number of viable cells in the disc which in turn lead to degeneration.[34]

To study the influence of inappropriate loading on IVDs, we recently investigated the effects of high frequency loading on discs in whole organ culture, which resulted in a significant drop in cell viability compared to discs that were

kept under physiological conditions.[27] Similar results were observed by Yamazaki et al.[35] by culturing rabbit AF cells for six days in monolayer. On the last day, cells were stimulated with a frequency of 6 Hz for 2, 4, 6 and 8 h. They observed a decrease in gene expression for extracellular matrix molecules and matrix metalloproteinases. Alternatively, Kasra *et al.* described a drop in DNA amount, impaired protein synthesis and increased degeneration at a frequency range of 3–8 Hz when culturing porcine IVD cells in alginate beads under dynamic hydrostatic loading at with an amplitude of 1 MPa.[36]

In the present study, BMSC adduction of metabolically and mechanically challenged bovine IVDs has been observed after 2-week culture. Compared to IVDs cultured under "simulated-physiological" conditions, the number of BMSCs that migrated towards the center of the disc was increased in "degenerating" IVDs; further, media of "degenerating" cultures revealed stronger chemo-attractive activity towards hBMSCs compared to "normal" cultures. By culturing bovine discs without endplate, the culture condition has been modified compared to the previous studies; which probably led to the observed alteration in the disc dimensions. The changes tended to be more pronounced when cultured under physiological loading. It is likely, that the applied low frequency might have accelerated the fluid exchange within the disc, whereas, high frequency load, similar to a static loading situation, may have even slowed down this process (for review see Iatridis et al.[37]). In dependence on a study of Lee et al., viable cells were observed after two week culture.[38] Lee reported that cell viability was maintained for seven days when culturing bovine IVDs without endplate under static loading, but biosynthetic activity of the cell dropped already after two day culture. In the present study the major question did not address detailed differences in the metabolic activity compared to fresh discs but focused on the chemo-attractiveness of stressed disc cells.

It has been described, that a partial degradation of the ECM is necessary to allow the migration of BMSCs into tissues.[20] Such degradation is often induced by MMPs,[22,39,40] proteolytic enzymes that are up-regulated by inflammatory

cytokines such as TGF-β1, IL-1β and TNFα[20]. In our previous study we observed an increased MMP13 gene expression in stressed discs.[27] This might be an indication that the early response towards the degenerating culture results in increased release of cytokines and MMPs and therefore promotes the homing of BMSC into the disc. A limitation of this study is that we did not analyze the differences in gene expression of bovine discs without endplate, but additional experiments are planed to close this gap.

Migrated BMSC were not only observed in IVDs with "stressed" pre-culture but also after "normal" pre-culture and even in fresh discs (without pre-culture). A certain percentage of engrafting into the IVD probably result from the harvesting procedure, since all discs experience a basal injury while cutting away the EP and the surrounding tissues. However, one could argue that the relative BMSC enrichment within the "stressed" discs, especially in those that were stabbed with a needle, is due to the manipulation that those IVDs experienced. To verify this possibility, four discs were frozen at -20°C to deplete them from native cells, and then dead tissues were punched, pre-cultured under high frequency load and co-cultured like the other groups. The rate of BMSC migration into this dead tissue was similar to the migration into freshly harvested discs and slightly lower compared to the BMSC engraftment towards discs pre-cultured under simulated-physiological conditions. This indicates that the 7 days pre-culture may already induce a certain level of mediators leading to increased cell attraction. Furthermore, this observation confirms that the BMSC recruitment is a cell-mediated effect that cannot be observed with cell-depleted discs.

To further analyze whether BMSC homing was mediated by chemotactic factors, we compared cultures of BMSCs co-cultured with conditioned media from "normal" and "stressed" discs. The experiments demonstrated a two-fold increased chemotaxis of the "stressed" media over the "normal" media, indicating an early response of the suffering discs. Further experiments will be required to identify the signaling factors responsible for this chemotactic activity. Although to date the presence of chemokines has not been documented in degenerating disc,

herniated disc tissues are known to express chemotactic molecules that particularly attract cells responsible for tissue resorption such as macrophage.[41] On the other hand, chemokine receptors have been identified on human BMSCs and the in vitro migration capacity of BMSCs in response to various chemokines and growth factors has recently been described.[42]

The main limitation of this work is that the organ cultures were preformed without endplates, since it is known that this affects the composition of the ECM going along with alterations of the native environment of the disc cells. It additionally remains questionable whether the BMSCs would be still capable to migrate into the disc center if the IVD would include endplates. This part of the current study addresses the potential of BMSC attraction by stressed IVDs. Looking at sagittal sections, we could observe that migration into the disc center occurred not only from cranial and caudal directions, but also through the outer AF. Based on this finding, further investigations will include BMSC homing in IVDs with endplates and focus on metabolical factors that are likely to stimulate BMSC migration.

Taken together, to our knowledge, we are the first to demonstrate in an organ culture that early degenerating discs can release factors that can induce MSC homing. Several recent studies have shown that human BMSCs have the capability to survive within the disc. Injection of human BMSCs into injured porcine spinal discs,[43] a rat disc degeneration model,[44] and bovine caudal discs in vitro[45] demonstrated BMSC survival and differentiation towards a disc-like phenotype. Moreover, recent work indicates that human degenerate discs contain populations of skeletal progenitor cells.[46] Additionally, Henriksson *et al.* detected progenitor cells, cell proliferation zones and potential stem cell niches within the IVD of rabbits, minipigs, rats and humans.[47] Our results contribute to these observations and may have implications for IVD regenerative treatment such as recruitment of endogenous progenitors or delivery of exogenous cell populations.

In the near future, the scheduled experiments will be finalized. In general, the number of replicates needs to be increased. Moreover, the underlying trophic factors for IVD homing of BMSCs will be investigated in more detail and the identified chemo-attractant molecules will be isolated for further investigations. Additionally, the potential of chemo-attraction will be investigated in intervertebral discs with endplates in a long-time co-culture with BMSC to study the homing capability of BMSC through the EP. Beside this, further experiments with chondrocytes and fibroblasts are planned. To investigate whether the homing of cells into the IVD is specific for BMSCs, we will co-culture stressed IVDs with chondrocytes and fibroblasts. We hypothesize that BMSCs but not other cell types will be attracted by chemokines that are released by the stressed discs.

6.6 Acknowledgement

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Chapter 7
General discussion

7.1 Summary

The primary purpose of this thesis was to establish a novel *in vitro* whole organ culture system to maintain whole intervertebral discs with endplates in mid-term culture and further study the effects of declined nutrition supply and inappropriate frequency loading on disc cells. The latter particularly with regard to the combined effects of limited nutrition and high frequency load since it is assumed that subjects that are already predisposed for developing disc degeneration (DD) e.g. due to nutrition shortage of the IVD, are likely to be more prone to develop DD when exposed to high frequency loading.

The reproducible model for degenerating discs was further used to investigate the homing potential of bone marrow derived mesenchymal stem cells (BMSC) in degenerating intervertebral discs (IVD). The establishment of the culture system is presented in chapters four and five. At the start of this project, there was no *in situ* whole organ culture system available for investigating disc degeneration. In the first part of this thesis, the influence of limiting nutrition in a mid-term culture was investigated. When cultured with media containing "sufficient" concentration of glucose, cell viability and matrix synthesis at the gene expression level were maintained for up to three weeks. Whereas, reducing glucose concentration caused increased cell death in the disc culture system with a drop in cell viability to ~ 50%. This finding confirmed Horner & Urban's (2001) *in vitro* cell culture work on the effect of low glucose conditions on disc cells.[1] However, besides decreased cell viability, no effects on cell metabolism could be observed. Based on those results, the effect of high frequency loading, simulating whole body vibration, was investigated additionally. Exposure to a frequency of ~10Hz is experienced when flying in a helicopter[2] and therewith similar to the natural vibration frequency in a human body[2]. In the whole organ culture, high frequency load had a negative effect on cell viability and the combined culture under limiting nutrition and high frequency increased the cell death even further. Moreover, a response in gene expression was observed in the stressed discs. In

chapter six, the degenerating model was used to investigate whether metabolically and mechanically challenged discs release factors that attract human BMSCs. Adduction of BMSCs was observed after two-week culture. Compared to IVDs cultured under "simulated-physiological" conditions, the number of BMSCs that migrated towards the center of the disc was increased in "degenerating" IVDs. Conditioned media of "degenerating" discs also demonstrated chemo-attractive activity towards hBMSCs.

The results compiled in this thesis present the development of a whole organ culture system for investigation of both degenerative as well as regenerative processes of the intervertebral disc.

7.2 Relevance of whole organ culture models

Disc degeneration often begins in the second decade of life and increases with aging.[3] Both limited nutrition[4] and inappropriate load[5] can lead to alteration of cell viability and disc functionality. Therefore, there is an increasing need to better understand the processes of disc degeneration and to develop novel regeneration strategies. Recently, several approaches to induce disc degeneration in different models have been published.[6-8] Often, degeneration is induced by needle puncture[9] or chemical factors like trypsin[10] or chymopapain[6] injection. There is no doubt that partial digestion of the disc matrix is suitable for creating a cavity, since therapeutic agents for regeneration of the intervertebral disc are often injected directly into the disc. However, enzymatic digestion might not always be necessary for creating degenerating IVDs. One should not disregard that this treatment significantly alters the ECM of the disc. An almost complete loss of GAG from the matrix within a short time would not be an exact replication of what happens in the degenerating disc. Naturally, there are no extracellular chondroitinases present in mammals, a circumstance that for some questions should be kept clearly in mind. The results presented in this thesis provide not only a culture system that is capable for maintaining intervertebral disc cells in

their native environment but also gives a deeper insight into the biology of degenerating discs. Even though severe disc degeneration cannot be achieved with this culture system, the present model is a feasible system for reproducibly creating degenerating discs that can be directly compared to discs that were cultured under physiological conditions. The minimization of the error probability for misinterpreting culture artifacts by comparing degenerating discs not only to fresh discs is one of the advantages of the presented culture system. Finally, the last part of the thesis is based on the degenerating system and aims to investigate the biologic restoration of the early stages of IVD degeneration. Healthy disc cells from a patient are difficult to obtain and limited in number. The autologous transplantation would require more cells than present in a single healthy disc, and harvesting NP cells from healthy discs could lead to their degeneration. As a consequence, the focus for donor cell-transplantation has shifted from autologous NP cells to stem cells. Since the first report of BMSC transplantation into the IVD, several animal studies have confirmed the effectiveness of this procedure.[11,12] BMSC therapy aims to restore or decelerate early stages of disc degeneration. However, injection of cells or drugs into the disc goes along with puncture of the NP, which in turn could induce disc degeneration itself. The results of this thesis suggest that during early stages of degeneration IVD cells show chemo-attractive activity towards BMSCs.

7.3 Clinical significance

Due to its implication in low back pain, IVD degeneration is a widely investigated disorder. Current treatments involve conservative care like medication and physical therapy. If the well-being cannot be restored, surgical intervention is performed to obtain relief of pain. This includes spinal fusion, total disc or NP replacement.[13,14] However, those methods focus on relief of pain without addressing the underlying cause. Hence, in the effort to better understand disc degeneration and find biological therapies, *in vitro* and *in vivo* approaches to

simulate disc degeneration have been considered. Because of the difficulty of obtaining human tissues, especially healthy human discs, culture systems for animal models have been developed. However, many of those studies have used discs from animals that contain notochordal cells, or have cultured IVDs under free swelling conditions. Both approaches are known to deviate from normal human IVDs. As shown in this thesis, whole organ culture of ovine IVDs can be performed for up to three weeks and "normal" as well as "degenerating" discs can be obtained reproducibly. As shown in the second part of this thesis, this culture system is a suitable model for investigating regenerative approaches for IVD restoration. Moreover, the whole organ culture provides an important link between conventional *in vitro* and *in vivo* experiments. As such it can make a significant contribution for reducing the use of live animals in research to a minimum, reducing *in vivo* animal models as far as possible.

7.4 Limitations

A general limitation of all animal studies is that they may miss the importance of genetics, which is believed to contribute up to 75% to disc degeneration in humans.[15,16] However, including genetics in animal studies is still a problem, since the participating genes are not known yet. In a recent study, Sakai *et al.*[17] found considerable interspecies differences when comparing gene expression levels of notochordal and non-notochordal animal discs with human IVDs.

Another limitation is the use of tail IVDs, since they cannot be considered to be weight bearing in the same manner as human discs. Like in most tails, ovine and bovine caudal IVDs are mainly loaded through contraction or tension of passive structures. The more the muscles contract and passive structures become tensioned, the more the loading of the tail discs will increase. However, measurements of the in vivo loading of the tail are not available yet. Therefore,

the influence of the mechanical environment on the behavior of disc cells is not known.[18]

A further limitation is the restriction in disc size. With 20 mm in diameter, the bioreactor chamber can be loaded with discs that have a maximal diameter of 15 mm to ensure media distribution around the disc. For the work of this thesis ovine and bovine IVDs of a range from 8-15 mm were used. Even though slightly smaller than human discs, the thickness (5-10 mm) of discs from both models is similar to human. The musculature of the bovine tail maintains an in vivo pressure on the discs that is similar to the human lumbar disc in the prone position (0.1–0.3 MPa).[19]

It is known that disc cells have to survive in a harsh environment, experiencing hypoxic and relatively acidic conditions.[1] Those parameters could not be included in this thesis, since the setup of the bioreactors did not provide the possibility to measure the oxygen tension or pH concentration within the disc. However, the organ culture model presented in this work provides the vantage of culturing the entire IVD. Therefore, nutrition, oxygen and metabolic waste products have to diffuse through the disc, a setting similar to the *in vivo* situation. Thus, it can be assumed that the relative composite of those parameters equates to the environment found *in vivo*.

Due to the declined availability of adult ovine caudal IVDs and the disproportion of performing euthanasia just for this study, bovine IVDs that were kindly provided by the local butcher were used for the second part of this thesis. Since the aim of the study was to investigate if BMSCs are attracted by factors that are released from the stressed disc cells, the cultures for this study were preformed without EP. Under this condition, migration towards the disc centre is likely to be better accessible for BMSCs. In fact, it is known, that if cultured without EP, discs swell markedly and lose glycosaminoglycans, thus changing the extracellular environment. However, one of the features of the described bioreactor system is to apply load in a physiological manner,[18] therefore swelling

of the IVD was avoided. Additional degenerative changes in the ECM of the disc, resulting from the removal of the EPs, in turn might lead to further release of chemo-attractants. This could be an additional explanation for the observed basal homing rate of BMSCs into the disc. Since, at this point of the study, the main emphasis was not placed on changes of the ECM, the impact on tissue properties was not assessed specifically. Nevertheless, in the advanced study, the IVDs will be cultured with EPs.

Another point that was not considered so far concerns the potential changes of the migrated BMSCs. Therefore, it is planed to further explore the proliferation and the differentiation potential of the migrated BMSCs into NP like cells. Additionally, the effect of BMSCs on NP cells could be examined. It would be of high interest to ascertain whether BMSCs are capable to activate NP cells in the presented *ex vivo* whole organ culture in a way that would be comparable to *in vitro* studies.

It is known that structure and composition of the IVD are changing with aging. Therefore, the age of the animals, respectively their discs, is relevant. Sheep used for the work were skeletally mature. However, due to limitations in disc size, only tails from calf in the age of 7-8 months were used.

7.5 Conclusion

The explant culture system presented in this thesis is the first that confirms the finding from Urban *et al*.[20] that the reduction of nutrition has a negative effect on the cell viability of the IVD. Moreover, high frequency loading simulating vibration conditions shows detrimental effects on IVD cells in whole organ culture. The effect was exacerbated by limited nutrition culture. However, we cannot predict, whether frequency or limited glucose affected cell metabolism.

But, what does this indicate? Are there no degenerating processes ongoing? And, what is generally meant by “disc degeneration”? Recently, Adams and Roughley proposed the following definition: “The process of disc

degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerate disc is one with structural failure combined with accelerated or advanced signs of aging. Early degenerative changes should refer to accelerated age-related changes in a structurally intact disc. Degenerative disc disease should be applied to a degenerate disc that is also painful".[21]

Therefore, the results presented here implicate that, with this system, degenerating but not degenerated discs can be generated under the current conditions. Nevertheless, it is conceivable that if the cells were given more time to respond to the changing conditions, e.g. by increasing the culture time, one could expect a stronger response also on ECM level. Therefore, further experiments will investigate effects of extreme high (10Hz) frequency loading and varying nutrition in a long-term culture, since the process of DD is a long-term process. Nonetheless, the current system provides the opportunity to investigate regenerative strategies on metabolically and mechanically challenged IVDs. Already after 2-week culture, BMSC homing into the disc center was increased in "degenerating" cultures compared to "normal" cultured IVDs; further, media of "stressed" discs revealed stronger chemo-attractive activity towards hBMSCs compared to "normal" cultures. Since regenerative effects of attracted BMSCs can be envisaged, this finding has implications for therapeutic delivery of BMSCs and endogenous progenitor cell activation.

7.6 Perspectives

The system for culturing the entire organ of larger IVDs described in this thesis might have great impact on future research on disc regeneration. This whole organ culture model provides the unique possibility to reproducibly create degenerating discs and thereby supplies the basis for evaluating the regenerative effects of growth factors, cytokines and protease inhibitors. Those factors can be introduced into the IVD by direct injection or application through the media. The impact on the metabolic activity of disc cells can be investigated in a controlled

environment that is nearly identical to *in vivo* conditions. This model can also be used to evaluate tissue engineering approaches that are designed to treat disc degeneration, like the implantation of cells, either alone or embedded in a scaffold. Even though in vivo experiments will be necessary also in future, the use of bioreactors as described in this thesis might contribute to reducing the use of live animals in research.

7.7 References

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Appendix

List of figures

List of abbreviations:

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AF	annulus fibrosus
AFC	annulus fibrosus cells
ASPN	Asporin
BMSC	bone marrow derived stem cells
CEP	cartilaginous endplates
CILP	cartilage intermediate layer protein
COL	collagen
CS	chondroitin sulphate
DD	disc degeneration
DDD	degenerative disc disease
DMEM	Dulbecco's Modified Eagle Medium
ECM	extra cellular matrix
EP	endplate
G	globular domain
GAG	glycosaminoglycan
GF	growth factor
GFP	green fluorescent protein
IL-1	interleukin 1
IVD	intervertebral disc
KS	keratan sulphate
LBP	low back pain
MMP	matrix metalloproteinase
NDR	nucleus pulposus replacement
NP	nucleus pulposus
NPC	nucleus pulposus cells
OP-1	osteogenic protein-1
PBS	phosphate buffert saline
PG	proteoglycan
SLRP	small leucine-rich proteoglycan
SNP	single nucleotide polymorphism
TDR	total disc replacement
TGF-β	transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TNFα	tumor necrosis factor alpha
WBV	whole body vibration

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List of Publications

Articles

1. **Jünger S**, Gantenbein B, Alini M, Ferguson SJ and Ito K.: Effect of limited nutrition on in situ intervertebral disc cells under "physiological" loading. Spine 2009;34:1264-71

2. **Illien-Jünger, S**; Gantenbein-Ritter, Grad, S; Lezuo, P; Ferguson, S J; Alini, M and Ito, K. The combined Effects of Limited Nutrition and High Frequency Loading on Intervertebral Discs with Endplates. Spine (accepted)

3. **Illien-Jünger, S**; Grad S, Sakai D, Mochida J, Alini M, Homing of Mesenchymal Stem Cells in stressed Intervertebral Discs in Organ Culture. Spine (to be submitted)

Awards

4. 1st place in the Student Oral Presentation; ECM IX; 15-18 June 2008, Davos **Juenger S**; Gantenbein B; Alini M; Ferguson S.J; Ito K; Effect of limited nutrition on Intervertebral Disc cells under “physiological” loading – a 21 day culture

5. Young Researcher Award 2009, AO Spine, Europe (AOSEU) **Illien-Jünger, S**; Gantenbein-Ritter, Grad, S; Lezuo, P; Ferguson, S J; Alini, M and Ito, K The combined effects of limited nutrition and high frequency loading on intervertebral discs with endplates

Oral presentations

6. **Jünger S**, Gantenbein B, Lezuo P, *et al.*: The combined effects of high Frequency Loading and Limited Nutrition on Intervertebral Discs Orthopaedic Research Society (ORS); Las Vegas, NV, USA, 22-25 February, 2009 (Podium presentation)

7. **Jünger S**, Gantenbein B, Lezuo P, *et al.*: Effect of limited nutrition on intervertebral discs under cyclic loading in a seven day culture, Orthopaedic Research Society (ORS); San Francisco, CA, USA, 2-5 March, 2008 (Short talk)

8. **Jünger S**, Gantenbein B, Lezuo P, *et al.*: Effect of limited nutrition on intervertebral disc cells under cyclic loading in a whole organ culture, Spine week, Kyoto, Japan, 23-26 January 2008 (Podium presentation)

9. **Illien-Jünger S**, Grad S, Sakai D, *et al.*: Homing of mesenchymal stem cells in stressed intervertebral discs in organ culture, International Society for the Study of the Lumbar Spine (ISSLS), Auckland, New Zealand 13-17 April, 2010 (accepted for podium presentation)

Posters

10. **Jünger S**, Grad S, Gantenbein-Ritter B, Grad S, Lezuo P, Ferguson, Alini M, Ito K: The Combined Effects of high frequency loading and limited nutrition on Intervertebral Discs with Endplates, Swiss society for Biomedical Engineering (SSBE), Bern, Switzerland, 27-28. August 2009

11. **Jünger S**, Grad S, Gantenbein-Ritter B, Lezuo P, Ferguson, Alini M, Ito K: Combined effects of limited nutrition and high frequency loading on intervertebral discs, International Society for the Study of the Lumbar Spine (ISSLS), Miami, FL, USA, 4-8 May 2009

12. **Jünger S**, Gantenbein B, Lezuo P, et al.: Intervertebral Discs and the Effect of limited nutrition – a 21 day culture, Swiss society for Biomedical Engineering (SSBE), Basel, Switzerland, 4-5. September 2008

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14. **Jünger S**, Gantenbein B, Lezuo P, et al.: Effect of limited nutrition on in situ intervertebral disc cells under "physiological" loading, International Society for the Study of the Lumbar Spine ISSLS), Hong Kong, China, 10-14 June 2007

Declaration of Originality

Last name, first name: **Illien-Jünger, Svenja**

Matriculation number: **06-117-766**

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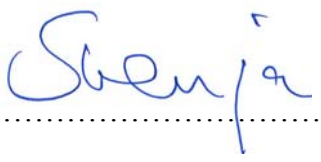
17.03.2010	
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