POST-TRAUMATIC INTERVERTEBRAL DISC DEGENERATION – AN IN VITRO STUDY ON THE ETIOPATHOGENESIS

A B H A N D L U N G zur Erlangung des Titels

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Fracture of the vertebral endplates but not equienergetic impact load promotes disc degeneration in vitro

Severity and pattern of posttraumatic intervertebral disc degeneration depends on the type of injury

Early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent disc in vitro

Splenic mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro
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Zusammenfassung


Degeneration of the intervertebral disc is of great socioeconomic importance, as it is a principal factor for chronic low back pain and impairment of spinal function. The degenerative process is associated with an aberrant cell-mediated response to structural failures of the endplate and the disc. Failures either occur slowly and progressively (‘idiopathic’), due to genetic predisposition, adverse occupational loading, and smoking, or acutely by traumatic events, such as burst fracture of the endplate/vertebra. However, current treatments target clinical symptoms, instead of the underlying degenerative processes, because these degenerative processes are poorly understood. The investigation of the pathogenetic mechanism is challenging, as no genuine disc degeneration (DD) model exists which reproduces clinical occurrences. Recently, an in-vitro burst fracture model was established, resolving this limitation. Based on this model, the etiology and the pathobiological processes of post-traumatic DD have been investigated and interpreted in relation to ‘idiopathic’ DD.

Using a further-developed version of the in-vitro model for post-traumatic DD, structural damage has been identified as the pivotal factor driving the degenerative process. The findings demonstrate that fracture of the endplate has multiple adverse effects; first, it immediately boosts degenerative processes in the disc, second, it inevitably leads to accumulative damage of the disc under post-traumatic loading, and third, it facilitates an auto-immune response by co-localizing expelled nucleus pulposus material with immune cells from the bone marrow.

In conclusion, this thesis provides a better understanding of the post-traumatic degenerative process in the disc and points out that post-traumatic loading and immunological aspects can aggravate this process. Although the pathogenesis of DD depends on the etiology, ‘idiopathic’ and post-traumatic DD share the same characteristics, namely inflammation, apoptosis, and matrix resorption. Therefore, the burst fracture model and results from this model facilitate the development of treatment approaches for ‘idiopathic’ and post-traumatic DD, which target the degenerative process and not clinical symptoms.
Chapter 1

Introduction
1.1 Motivation

Low back pain (LBP) accounts for 75-85% of total workers’ absenteeism\(^1\) and costs more than 100 $ billion per year in the U.S\(^1,2\). Up to 84% of the population suffers from LBP at least once in their life, most frequently between the ages of 35 and 55. Approximately 23% complain about chronic LBP (more than 6 months’ duration) and 11-12% are disabled by LBP. There is no doubt that disc degeneration (DD) is involved in most of these cases, either as a primary pathology or as sequel of e.g. disc herniation or burst fracture\(^3,4\). MRI imaging is a reliable diagnostic tool for the identification and grading of degenerated intervertebral discs (IVDs)\(^5,6\). Although a degenerated IVD is not necessarily painful, pain correlates with the degree of degeneration\(^7\).

The socioeconomic importance of LBP and the clinical relevance of DD are recognized and promote extensive disc research. Nonetheless, to date, LBP cannot be treated effectively in the long-term. Since the exact etiopathology of DD is still poorly understood, current treatment strategies target clinical symptoms instead of the underlying pathological mechanism that causes DD. However, in the last three decades, it has become clear that DD is a multifactorial disease with a large hereditary factor and many associated life-time risk factors, mainly nutritional aspects and excessive mechanical loading.

From studies on human cadaveric tissue, we know many of the pathobiological characteristics of DD. Inflammatory, apoptotic, catabolic, and aberrant anabolic processes proceed in parallel and compound progressive degenerative changes\(^8-11\). The precipitating factor for the onset of these changes is damage or microdamage of the endplate\(^8\). Structural impairment of the endplate is also a key indicator for a painful IVD\(^12\). It is therefore not surprising that other pathologies of the endplate, like burst fractures\(^3,10\), Modic changes\(^13,14\) and Schmorl’s nodes\(^15\), are associated with DD and LBP.

Setting up a clinically relevant model for a mainly “idiopathic” disease is challenging. With simplistic models of cultured disc fragments, disc cells in monolayers or embedded in scaffolds, knowledge about basic disc cell metabolism has been gathered. However, the study of DD with these models is strongly limited, as the cells are devoid of their natural environment, which causes dedifferentiation and cell death\(^16-18\). In contrast, animal models provide reliable insights into the pathogenesis of DD, as the native IVD structure is preserved\(^19\). Their drawback, on the other hand, is that DD has to be induced artificially. Current methods of DD induction are digestion of the NP by protease injection, static overload, endplate perforation, or laceration of the annulus fibrosus (AF)\(^20-23\). Since most of these methods do not represent common occurrences in living people, they impose a strong limitation on the investigation of how degenerative changes are initiated\(^8\). During the last
decade, organ cultures of IVD explants, with or without endplates, were developed\textsuperscript{24}. Using a bovine tail explants model, DD was successfully induced by nutrient deprivation combined with hyperphysiological loading\textsuperscript{25}. Although, this model represents a clinically relevant scenario, the subsequent degenerative processes cannot be investigated, due to the available culture period, which is limited to a few days to weeks.

Accumulating clinical evidence for the association of DD with burst fractures of the vertebra/endplate showed that DD is not only an “idiopathic” disease, but can also be triggered by a clearly defined event\textsuperscript{26,27}. Therefore, a burst fracture represents a suitable model for the investigation of the etiological factors of DD. Although acute trauma and “idiopathic” DD have different etiologies, their pathobiological courses seem to correspond phenomenologically to a great extent\textsuperscript{10}. However, until recently, the investigation of post-traumatic DD was hampered by obvious ethical concerns to induce burst fractures in-vivo. Consequently, post-traumatic DD is still sparsely investigated and little is known about the acute biological response of the IVD to high velocity impact loading and/or endplate trauma and its correspondence to non-traumatic DD. A key achievement in this field was the establishment of the in-vitro burst fracture model using whole rabbit IVD/endplate explants\textsuperscript{26}. Using this model, Haschtmann et al. showed for the first time that experimental burst fracture indeed triggers DD\textsuperscript{26}.

This model opened new ways for the systematic investigation of post-traumatic DD, in particular the identification of the etiological factors, the pathogenetic mechanism, and the implication of the immune system. Considering the results from disc herniation studies, the initial post-traumatic degenerative changes must be comparatively complex and numerous pathobiological changes and inflammatory cascades, such as induction of apoptosis, activation of matrix-metalloproteinases (MMPs), and leukocyte recruitment, are likely involved\textsuperscript{23,28–30}.

Therefore, our motivation was in a first step to further develop the Haschtmann model and to make it suitable for the investigation of the etiopathology of post-traumatic DD. In a second step, we aimed to identify the etiological factors triggering the acute degenerative changes after burst fracture and to compare the pathogenetic response of the IVD to different types of trauma. The third aim was to investigate whether post-traumatic physiological loading represents a secondary trauma, which aggravates the degenerative process. In a last step, we investigated whether burst spinal segments have the intrinsic ability to evoke an autoimmune response when co-localized with leukocytes.
1.2 Thesis outline

In chapter 2, more detailed background information on the anatomy of the IVD and the basic principles of IVD biomechanics is provided. Furthermore, the current pathobiological knowledge about DD and its treatments is summarised. A subchapter is dedicated to the significance of the immune system in DD. The chapter ends with the discussion of current models for the investigation of DD and the presentation of the burst fracture model used throughout the thesis.

Chapter 3 to 7 comprise the main body of work of the thesis and each represents an unmodified manuscript, published in or submitted to peer reviewed journals. Their objectives and hypotheses are summarised in the following section. In chapter 8, the findings and limitations are discussed in a general context. The thesis closes with a conclusion and outlook in chapter 9.

1.3 Objectives and hypotheses

The overall goal of the thesis was to investigate in-vitro the etiopathology of post-traumatic DD and the implication of the immune system in this process. This overall goal was split into five specific aims. Each of these was investigated in an independent study. Their objectives and hypotheses are outlined below.

Chapter 3: Prior storage conditions and loading rate affect the in vitro fracture response of spinal segments under impact loading

A biomechanical investigation of the in-vitro burst fracture model. This study was published 2011 in the Journal of Biomechanics 44: 2351–5.

The rabbit explant model developed by Haschtmann et al. subjects freshly harvested IVD/endplate specimens to high-velocity impact loading. It differs in three aspects from general biomechanical testing protocols of spinal segments. First, it uses fresh instead of frozen/thawed specimens, second, specimens were subjected to high impact loading instead of quasi-static loading, and third, specimens were devoid of adjacent vertebral bones and did therefore not qualify as spinal segments. A number of studies demonstrated that freezing/thawing cycles, a common occurrence when specimens have to be stored before the tests, did not influence biomechanical testing when quasi-static loading conditions were used, but the effects of high impact velocities remain unclear. In-vitro experiments confirm the pivotal role of impact velocities on the characteristics of the resulting spinal injuries.
In order to biomechanically validate our in-vitro model, we investigated the effects of freezing/thawing and different impact velocities, by loading fresh and frozen/thawed cadaveric rabbit spinal segments with different impact energies and velocities using a custom-made, dropped-weight loading device. We tested the hypothesis that both parameters, prior storage condition and loading rate, affect the in-vitro fracture response.

Chapter 4: Fracture of the vertebral endplates, but not equienergetic impact load, promotes disc degeneration in vitro

A characterization of post-traumatic pathobiological processes in the IVD. This study was published 2012 in the Journal of Orthopaedic Research 30(5):809–16.

It is accepted that burst fracture promotes DD. However the pathogenetic processes leading to this finding are insufficiently understood. In particular, the question of whether a single impact without structural impairment of the endplate, which represents a frequent trauma during a lifetime, is sufficient to promote DD was never addressed. The established model was utilized for the further description of the post-traumatic degenerative process and to test whether a single equienergetic impact load without vertebral fracture is sufficient to promote DD. The pathobiological response of the IVD was tracked by histology, by quantifying the loss of glycosaminoglycans (GAG), by measuring necrotic and apoptotic protein activity, and by quantifying different pro-inflammatory, pro-apoptotic, catabolic, and anabolic gene transcriptions.

Chapter 5: Severity and pattern of posttraumatic intervertebral disc degeneration depends on the type of injury

The identification of the triggering factor causing post-traumatic DD. This study has been re-submitted, following peer-review and revision, to The Spine Journal.

The etiopathology of post-traumatic DD is insufficiently investigated, thus the development of regenerative therapies is only slowly progressing. In order to treat the cause and not the symptoms, the identification of the etiological factors and the characterization of its pathobiological effects are pivotal. Burst fracture of a vertebra is the result of a complex loading procedure and is often associated with the degeneration of the adjacent IVD. Likewise, the presumed etiologies are numerous: (i) the structural perturbation of the IVD/endplate, (ii) the impact loading energy alone, and (iii) the depressurization of the nucleus pulposus. In order to investigate in detail the
contribution of each factor (i to iii) to DD, we extended our previous work described in chapter 4 and compared three different segmental trauma processes using the established rabbit full-organ in-vitro model. The three trauma were: burst fracture, (etiologies i-iii), equienergetic loading without a fracture (ii), and endplate puncturing (iii). We hypothesized that the structural perturbation of the IVD/endplate (etiology i), which is unique to the burst fracture model, is the major factor causing posttraumatic DD and that different etiologies cause different pathogenesis. DD was assessed by the same markers as in the project described in chapter 4.

Chapter 6: Early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent disc in vitro

An investigation of a common loading scenario on the course of post-traumatic DD. This study has been submitted to the Journal of Orthopaedic Research.

The optimal treatment of a stable burst fractures is a topic of debate. Both surgical intervention and conservative treatment with early mobilization are practiced. In any case, the choice of treatment is based on the instability of the vertebra and the degree of posture loss. However, burst fractures also disrupt the adjacent IVD and trigger acute degenerative changes. It was shown that secondary trauma can cause chronic inflammation and DD, but it is not known whether early physiological loading represents a secondary trauma. In addition, little is known about the mechanisms driving DD from an acute to a chronic response. Given the natural severity of burst fractures, the pathobiological response of the IVD is strong and requires weeks until normalization is achieved. Therefore, we hypothesized that even physiological (moderate) loading during the first 4 weeks after trauma can cause a persistent deterioration of disc metabolism and enhance DD. We addressed these hypotheses by studying the response of the IVD to physiological loading after burst fracture using our establish in-vitro burst fracture model. For the post-traumatic physiological loading, a novel multi-specimen loading station was designed.

Chapter 7: Splenic mononuclear cells are attracted to and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in-vitro.

An investigation of the autoimmunogenic properties of the IVD. This study is ready for submission.

The healthy IVD is an immune-privileged organ. This privilege occurs by the avascular and alymphatic structure and the expression of fas ligand.
INTRODUCTION

Burst fractures compromise this privilege and NP material is expelled into the vertebral body, which leads to the co-localization of NP cells and matrix fragments with immune cells of the vertebral bone marrow. From disc herniation studies there is evidence that the NP is recognized by leukocytes as “foreign” and that an auto-immune response is triggered\(^{10-43}\). However, there is still uncertainty as to which compound is immunogenic and as to which are the mediating cell types and cytokines\(^{42,44-48}\). We hypothesized that a similar immune response occurs after a burst fracture. Therefore, the aim of this study was to elucidate, by using simple in-vitro models, if structurally impaired IVDs in general, and extracellular matrix (ECM) fragments and NP cells in particular, can attract and activate leukocytes. Rabbit splenic mononuclear cells (MNCs) were exposed to burst rabbit spinal segments, NP ECM fragments, or NP cells. Chemotaxis and activation of MNCs was quantified.

1.4 References


Chapter 2

Background
2.1 Anatomy of the human intervertebral disc

The human spine consists of 34 vertebrae. From cranial to caudal there are 5 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 4 coccygeal vertebrae. The cervical, thoracic and lumbar vertebrae are articulating, the sacral and coccygeal vertebrae are fused. Lordotic curvature in the cervical and lumbar spine and kyphotic curvature in the thoracic and sacral/coccygeal spine provide the spine with a “double-S” shape in the sagittal plane. Each vertebra is composed of a posterior neural arch and an anterior vertebral body (Figure 1a). The neural arch and vertebral body encircle the vertebral foramen, which houses the spinal cord. The vertebral bodies are roughly elliptical in shape. Two adjacent vertebral bodies enclose a fibrocartilage pad, the intervertebral disc (IVD).

The IVDs are the major mobile joints of the spine. They appear kidney shaped in the transversal plane and consist of three distinct structures: the central gelatinous nucleus pulposus (NP), the collagenous annulus fibrosus (AF), which surrounds the NP circumferentially, and the cartilage endplates (CEP), which separate the AF and NP from the vertebral bodies (Figure 1b and c). The IVD sided limits of the vertebral bodies are referred to as vertebral or bony endplates. They are composed of a layer (0.6 – 1 mm) of semi-porous thickened cancellous bone and form together with the CEPs the endplates (EPs). The IVD is comprised of an extensive extracellular matrix (ECM), which is maintained by cells with a tissue specific phenotype. They occupy less than 0.5% of the tissue volume.

![Figure 1. Schematic drawings of a human vertebra and IVD. (a) Transversal view of a lumbar vertebra. (b) IVD viewed from an anterior angle with partially removed AF lamellae. (c) Spinal segment consisting of two adjacent vertebrae and the interjacent IVD in a coordinate system indicating typical motions.](image)

2.1.1 Extracellular matrix (ECM)

The main function of the IVDs ECM is, on the one hand, to provide articulation, but also to provide resistance to the different spinal motions.
The three anatomical compartments of the IVD have evolved different microstructures in response to these tasks. The basic biochemical components of the NP, the AF and the CEP are the same, namely water, proteoglycans (PG) and collagens, but their relative proportions vary\(^4\) (Table 1). The ECM composition changes gradually along the radial axis with the central NP having the highest water/PG and lowest collagen/elastin content and the outer AF having the lowest water/PG and highest collagen/elastin content.

The CEP is an approximately 1 mm thick layer of hyaline cartilage. Its biochemical composition parallels the gradients formed by the NP and AF. In the central region, where the CEPs contact the NP, PG and water concentration is higher than in the more peripheral regions. Conversely, collagen concentration, mainly collagen type II, is lower in the central than in the peripheral region of the endplate. Collagen concentration also increases through the endplate thickness, towards the vertebral bodies.

<table>
<thead>
<tr>
<th></th>
<th>Nucleus pulposus</th>
<th>Annulus fibrosus</th>
<th>Cartilage endplate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>77%</td>
<td>60%</td>
<td>55%</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>14%</td>
<td>5%</td>
<td>8%</td>
</tr>
<tr>
<td>Collagen</td>
<td>4%</td>
<td>15%</td>
<td>25%</td>
</tr>
<tr>
<td>Elastin</td>
<td>&lt;1%</td>
<td>&lt;10%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Table 1. Approximate relative proportion of the four main components in the adult human IVD (adapted from Urban & Roberts\(^4\)). Generally PG and water decreases with age, collagen increases\(^5\).

The major PG of the IVD is aggrecan, a large molecule with a molecular weight ranging from 0.3 to 7.0 Mio Dalton. It consists of a core protein to which up to 100 keratan and chondroitin sulfates are covalently bound. These are highly negatively charged glycosaminoglycans (GAG), which imbibe water and confer a viscoelastic behavior to the tissue, in particular to the highly hydrated NP. The higher PG concentration in the NP, compared to the AF, results in higher water content and in a swelling pressure that is resisted by the AF and the EP. PG can bind to hyluronic acids (HA) via link proteins to form large aggregates. In the NP, only about 25 % of the PG are aggregated, in the AF 50-60 %. Aggregates are generally bigger in the AF than in the NP.

Collagen type I and II make up approximately 90 % of total IVD collagen\(^5\). The NP and the inner AF consist almost exclusively of type II. The relative proportion of type I collagen increases towards the outer AF\(^2,5\). Diameters of collagen fibrils are finer in the NP (ca 0.05 \(\mu\)m) than in the AF (0.1 – 0.2 \(\mu\)m)\(^4\). In the NP, collagen fibers are irregularly oriented; in the AF, they are organized into 10-25 uni-directionally aligned lamellae, which encircle the NP and attach to the CEP in the inner zone and to the vertebral endplates in the outer zone\(^6\) (Figure 1b). Alignment alternates between +30° and -30° with
respect to the circumferential direction of the IVD. The innermost lamella connects to the NP.

The most abundant non-structural proteins in the IVD are the families of MMPs (matrix-metalloproteinases) and ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs). These are zinc-dependent proteinases, which can cleave almost all components of the ECM.

2.1.2 Cells

The cells of the IVD synthesize and maintain the ECM. They control the homeostasis between ECM synthesis and degradation. Compared to other tissues, relatively few cells have to maintain an extensive ECM. Consequently, the turnover of ECM proteins takes years.

Three main types of cells are distinguished in the NP and AF: notochordal cells (NCs), NP and AF cells. NCs are remnants from the embryonic notochord and build the primary NP. They are significantly larger than NP and AF cells and contain vacuole-like cytoplasmic structures. In early childhood, NCs are replaced by NP cells. The AF and EP cells are of mesenchymal origin. According to their gene expression profile, AF cells are fibrocytic and NP cells chondrocytic. AF cells synthesize collagen I as the main structural protein, NP cells aggrecan and collagen II. Morphologically, NP cells are spherical and AF cells are elongated and aligned with the principal collagen fiber direction, but also stellate shaped AF cells have been reported. All three cell types sustain a pericellular matrix, similar to the chondron of chondrocytes, with a composition distinct from the intercellular matrix.

2.1.3 Nutrition

The IVD is the biggest avascular structure in the human body, with a distance of 6-8 mm from the cells in the center of an adult lumbar IVD to the nearest blood supply. Therefore, nutrient supply and drainage of metabolic products rely on diffusion between the IVD cells and the capillary endings of the vascular network in the outer AF and in the EP. The EP has a higher permeability in the central NP zone, which is essential to maintain a sustainable metabolite transport to and from the NP cells. The diffusion rate depends on the microstructure and composition of the transport route and on the biochemical properties of the solute (size, charge). Disc dehydration and endplate sclerosis impair diffusion, as they reduce the effective pore size of the ECM and block the EP, respectively. Impaired diffusion causes cell death and disc degeneration (DD) because of nutrient deprivation, mainly oxygen and glucose, and accumulation of metabolic products, mainly lactic acid (see 2.3.2.
Degenerative disc disease). Oxygen tension in the NP is anyway as low as 1% and its deprivation leads to a higher synthesis rate and accumulation of lactic acid, an anaerobic waste product, and consequently to a drop in pH. NP cells are maximally active at pH 6.9 – 7.2 but below pH 6.8 their activity is suppressed and they fail to retain a biomechanically sound ECM.

2.1.4 Innervation

The nerve supply to a healthy IVD is restricted to about the three outermost lamellae of the AF and to the central part of the EP. 90% of the nerves are sympathetic afferent and belong to the Sinus vertebral nerves (SNs). They are capable of sending nociceptive information to the sympathetic nervous system, which can cause a form of visceral pain similar to enteric structure. The SNs innervate up to three segments, which makes localization of low back pain difficult. Postganglionic sympathetic efferent nerves are in close association with the sympathetic afferent nerves and hence can sensitize them under certain conditions. This implies that psychological stress can cause a ‘central sensitization’, which lowers the threshold for IVD nociception and adds to chronic discogenic pain (see 2.3.4 Discogenic pain).

2.2 Biomechanics of the intervertebral disc

The IVDs are the main joints of the spine. Together with the surrounding ligaments and muscles, they provide articulation and transmit compressive forces from one vertebra to the next. They have evolved tailored shapes and microstructures to account for the various types of loading, like compression, bending, and torsion (Figure 1c).

Axial compressive loads are mainly resisted by the viscoelastic NP. Upon compression, its internal pressure increases and forces the AF fibers to bulge outwards. The fibers stretch, resist the tensile stresses and cause a circumferential stress at their site of attachment to the EP. This stress is also referred to as ‘hoop’ stress according to the steel hoops reinforcing a wooden barrel. Since annular bulging cannot accommodate the whole volume displaced, the EP is also forced to bulge into the cancellous bone of the vertebrae. Under axial overloads, it is generally the EP that fails before the AF (see 2.3.3 Post-traumatic disc degeneration).

Torsion causes the AF fibers of every second lamella to buckle and the fibers of the other lamellae to stretch and resist the force. Tensile stresses and strains increase with distance from the rotation axis and hence are biggest in the posterolateral sector of the AF and in the large and elliptically shaped
lumbar IVDs. Shear stresses also occur between AF fibers and in the transversal plane.

Bending results in stretching of the AF fibers and in tensile stresses on the convex side and in AF bulging and compressive stresses (caused by the body weight) on the concave side. The elliptical shape of lumbar and cervical IVDs helps to confer stability during flexion/extension while the cylindrical shaped thoracic IVDs are best adapted for torsion. Extension is mainly resisted by facet joints. Combinations of torsion and bending result in increased stresses and strains. With a superimposed load this can cause structural failure even in a healthy disc.

Loading also affects the fate and metabolism of the IVD cells. This occurs either directly by altering cell morphology or indirectly by altering the cells physical and biochemical environment. Type, magnitude, and duration of motions seem to play decisive roles. In the past years, bioreactors became increasingly popular to investigate the relevant parameters. Results of compressive load studies conflict in part, but general tendencies can be identified. Whereas static compression is always detrimental, under cyclic compression, there seem to exist a window of specific frequency, load, and duration, which favors cell metabolism. Frequencies between 0.01 and 10 Hz, loads between 0.2 and 1.3 MPa and loading times no longer than one hour a day have beneficial, or at least not adverse, effects on disc metabolism.

2.3 Disc degeneration

2.3.1 The ageing IVD

During life, the IVD undergoes slow but progressive degenerative changes, starting in the EP and NP and progressing to the AF. In the first year of life, the vascularity of the EP wanes and later its permeability decreases. As a consequence, metabolite transport is impeded and IVD cells fall quiescent or even die, in particular in the NP. This may explain why NCs completely vanish in the first years of life. As NCs have a higher PG synthesis rate and a stronger anti-inflammatory metabolism compared to NP cells, their loss is proposed as the initiation of the subsequent degenerative processes. The remaining cells with reduced metabolic activity are unable to sustain a proper functioning matrix. Unfavorable environmental conditions or mechanical injury can even accelerate the degenerative processes. Consequently, PG and water content are highest in the young adults and decrease afterwards. In contrast, collagen content increases and more cross-links are formed, resulting in thicker, stronger and less degradable fibers. This allows for non-enzymatic
glycation, another type of cross-link that makes the fibers excessively stiff and reduces their capability to absorb energy. The reduced hydration and volume of aged NPs cause a shift of compressive load-bearing to the stiffened AF and therefore increase the propensity of AF injury, in particular under high velocity loading\textsuperscript{35}.

### 2.3.2 Degenerative disc disease

Age related changes of the IVD are mostly degenerative and an 80-year-old IVD is most likely degenerated. However, degenerated IVDs have been described also in young adults or teenagers\textsuperscript{31-36}. In this case of accelerated DD, we speak of degenerative disc disease (DDD). The essential difference of DDD to an aged IVD is its increased EP permeability and accordingly also metabolite transport \textsuperscript{35,37-38}. While the main risk factors for DDD are genetic inheritance, nutritional impairment, smoking, repetitive heavy lifting, and ageing\textsuperscript{39,40}, the precipitating cause is structural damage occurring from injury or fatigue failure\textsuperscript{30,41}. The risk factors mainly affect the IVD (micro-) structure and hence predispose for structural failure. Thus, the strong association of age with DDD\textsuperscript{42} can be explained as the sum of risk factors. Since structural damage has the decisive influence on the degenerative process, it is not surprising that endplate trauma\textsuperscript{43-45} and Schmorl's node\textsuperscript{46} show high associations with DDD.

Structural damage occurs first either in the EP or the AF. Therefore, two different phenotypes of DD exist: EP-driven and AF-driven DD\textsuperscript{47} (Table 2). Despite the different initial modes of structural damage, the two phenotypes may finally progress on a common pathway until complete failure.

In the EP-driven model, axial overload causes excessive bulging of the EP into the vertebral body, thus EP defects occur and pain arises from the EP or vertebral body. Prerequisite for EP bulging is a highly hydrated NP, which is normally restricted to infants and young adults. The NP escapes through the EP defect and causes reduced swelling and loading pressure on the inner AF. Consequently, the inner AF fibers collapse and AF tears grow in size and number under loading. A weakened trabecular structure underlying the EP may even allow for sufficient EP bulging and consecutive delamination of the inner AF fibers without EP fracture. In lumbar IVDs, the extra volume created by EP bulging is only a small percentage of the total NP volume. Therefore, pressure drop is small and delamination is less likely to occur than in the thoracic IVDs.

The AF-driven DD is described as the progression of radial fissures with concomitant radial protrusion of the NP. A disc prolapse is a possible endpoint. Radial fissures are caused by excessive shear stress in the AF fibers, which can occur by the combination of flexion and torsion. Flexion causes high shear
stresses in the posterior outer AF and torsion causes increasing shear stresses with increasing distance from the rotation axis. Therefore, the highest stresses are found in the posterolateral fibers of the outer AF and in the elliptically shaped lumbar IVDs. Indeed, this is the location where most disc herniations occur. Once fibers have ruptured, the remaining stress has to be resisted by fewer fibers, thus the risk for tear progression is high. Middle-aged discs still have high NP hydration, but already a stiffened AF, which makes them most vulnerable for AF-driven DD.35

<table>
<thead>
<tr>
<th>EP-driven DD</th>
<th>AF-driven DD</th>
</tr>
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<tbody>
<tr>
<td>Associated with EP defects</td>
<td>Associated with AF fissures</td>
</tr>
<tr>
<td>Upper lumbar and thoracic spine</td>
<td>Lower lumbar spine</td>
</tr>
<tr>
<td>Higher heritability</td>
<td>Lower heritability</td>
</tr>
<tr>
<td>Often before age 30 years</td>
<td>Rarely occurs before age 30 years</td>
</tr>
<tr>
<td>Moderate association with pain</td>
<td>Strong association with pain</td>
</tr>
<tr>
<td>Pain from EP/vertebral body</td>
<td>Pain from peripheral AF/nerve root</td>
</tr>
<tr>
<td>Caused by spinal compression</td>
<td>Caused by spinal bending</td>
</tr>
</tbody>
</table>

Table 2. Comparison between two DD phenotypes. Adapted from Adams & Dolan.47

The progression of DD can be classified by the Thompson grading into five pathomorphological classes. From grade zero to grade V, homogeneity of the NP decreases and number and size of annular tears increase.48,49

A structural damage to an AF or EP causes regions of low and high compressive stresses, both of which are known to inhibit IVD cell metabolism.50 Therefore, the ability of the few IVD cells to repair the disrupted matrix is further reduced by altered matrix stresses. The result is a vicious cycle of frustrated healing attempts and repeated re-injury.30,47 This cycle is mirrored by an altered gene expression profile in cells of degenerated IVDs; catabolic genes from the MMP and ADAMTS families are over-expressed and anabolic gene transcription is switched from collagen type II and aggrecan towards collagen type I, versican, biglycan, and decorin.51 Another characteristic of degenerated IVDs is the chronic expression of pro-inflammatory mediators, in particular TNF-α and IL-1.52 Whereas IL-1 contributes to matrix degradation, TNF-α elicits a pro-apoptotic43 and nociceptive54 function. The release of inflammatory mediators after tissue injury is inherent to almost all tissues and is required for tissue repair. However, a persistent injury stimulus in conjunction with low metabolite exchange rates, as in a degenerative IVD, facilitates the formation of a destructive chronic inflammation. Resolution of the chronic inflammation and tissue healing are impossible.
2.3.3 Post-traumatic disc degeneration

Compressive overload at a high impact velocity causes a distinctive fracture mechanism known as a ‘burst fracture’. Burst fractures account for up to 17% of all major spinal fractures\(^55\). The compressive load increases intradiscal pressure, which causes the EP to bulge into the vertebral body and finally to crack, thereby forcing NP material into the vertebral body at a greater rate than the contents of the body can be expelled. This leads to a pressurization and burst of the vertebral body thereby expelling vertebral bone fragments into the spinal canal\(^55-58\). The degree of spinal canal occlusion correlates with the loading rate\(^57\). Burst fractures typically occur at the thoracolumbar junction\(^55\), as the region forms a transition zone between the relatively fixed, kyphotic thoracic spine above, and the relatively mobile, lordotic lumbar spine below. This causes stress forces to concentrate upon the thoracolumbar vertebral column\(^59\).

Pain often arises immediately after burst fracture as the disrupted EPs and vertebral bodies are innervated\(^60\) and bone fragments impinge spinal nerves\(^61\). In addition, burst fracture can also cause pain in long term as it triggers DD\(^43,44\) (see 2.3.4 Discogenic pain). Compared to other forms of DD, a single high impact energy is sufficient to disrupt the EP and the vertebral body and thus can trigger DD as a single event also in a previously healthy disc. This makes post-traumatic DD an eligible pathology for the investigation of the pathomechanisms in DD.

2.3.4 Discogenic pain

DD is not painful per se, as pain perception requires the stimulation of nociceptive nerve fibers. In a healthy disc, innervation is restricted to the outer third of the AF but in degenerated IVDs, nociceptive nerve fibers and mechanoreceptors penetrate along radial AF fissures deep into the IVD, in highly degenerated IVDs even into the NP and give rise to discogenic pain\(^14,15,62\). Many of these nerves are in perivascular position, suggesting that they arise from granulation tissue growing into the degenerative IVD. Granulation tissue is rich of inflammatory mediators and can sensitize nociceptive nerves and cause mechanoreceptors into becoming pain afferent, resulting in chronic discogenic pain\(^14\).

Low back pain (LBP) has an extremely high life time prevalence of up to 84 %\(^63\). More than one third can be directly attributed to DD\(^64\). As painful discs are always structurally disrupted\(^62\) and show irregular stress concentrations\(^65\), secondary deteriorations are very likely and further increase the portion of LBP, which can be attributed to DD.
2.3.5 Current treatments of DD

Today, treatments of LBP caused by spinal stenosis or by DDD aim for a quick pain relief. In the case of spinal stenosis, canal clearance provides effective pain relief in the short- and medium-term but not in the long-term, because the affected IVD often degenerates and becomes painful again\(^4\). Current treatments for DDD range from conservative therapies, such as bed rest, anti-inflammatory medication, analgesia and physical therapy, to invasive strategies, such as epidural steroid injections, discectomy, spinal fusion and disc replacement\(^4\). However, their long-term outcomes are unpredictable and mostly unsatisfactory, since they target clinical symptoms rather than the degenerative cascades causing the symptoms. Therefore, novel strategies aim to regenerate the IVD. Three general approaches were developed which can be employed alone or in combination: cell therapy (injection of disc or stem cells), injection or implantation of tissue engineered biomaterials, and molecular therapies (injection of growth factors, gene therapy). Some treatment approaches are currently tested in clinical phase I\(^4\). In addition, novel imaging techniques help identifying painful discs and even allow describing the metabolic state of the IVD, which in turn permits to tailor treatments\(^6\).

2.4 The IVD and the immune system

Vascularization of the healthy IVD is restricted to the EP and the outer AF. Hence, the NP is excluded from immune surveillance and defines an immune privileged tissue\(^6\). Besides the avascularity, expression of fas ligand (FasL) by NP cells mediates this privilege\(^6\). In other immune privileged organs like the retina, testis, and brain FasL expression has been shown to induce apoptosis in invading activated T-cells by binding to their fas receptor. Intriguingly, degenerated IVDs show reduced FasL expression\(^6\) and neovascularization\(^6,70,71\) thereby compromising both mechanisms of immune privilege maintenance. From disc herniation studies it is known that, once the NP is exposed to the immune system, co-localized leukocytes are activated and an autoimmune reaction is evoked\(^670-78\). However, direct evidence of an autoimmune reaction in DD has not yet been demonstrated.

2.5 Models for the investigation of DDD

The complex etiopathology of DD challenges researchers to develop models which are as simple as possible but as comprehensive as necessary. The most apt model always depends on the topic of investigation. Three fundamentally distinct models were developed in the last two decades: in-vitro systems with cultures of isolated IVD cells in monolayers or in 3-dimensional (3D) scaffolds, in-vivo models, and explant organ culture models.
The simplistic monolayer model has the big advantage of a completely controllable environment. In a slightly more elaborated version, IVD cells are embedded in an artificial 3D-structure, in order to account for the deprivation of the native ECM structure. Nevertheless, cell dedifferentiation or cell death is commonly seen. Since mechanical aspects are pivotal in the etiology of DD, the utilization of in-vitro models of isolated cells are limited.

DD can be induced effectively in animal models, either enzymatically or mechanically. Mechanical induction is achieved by means of static or dynamic overload, by EP perforation or by AF laceration. In-vivo models proved especially valuable for the identification of mechanical risk factors. An interesting in-vivo model is found in the chondrodystrophic dog breeds, which develop genetically inherited DD at a defined age. However, the lack of healthy control IVDs and the uncertainty about the reason for the high rate of DD impose major limitations. In all animal models, the complexity of a whole organism makes it difficult to investigate the intricate metabolism and signaling cascades of IVD cells.

Explants of IVDs aim to combine the advantages of in-vitro and in-vivo models; the culture environment is largely controllable without losing the native tissue and organ structure. This allows a generally good reproducibility. Explant models are also favorable in terms of cost efficiency and ethical aspects, as no experiments are performed on living animals and a maximal use of tissue is achieved. Retaining the EP has been shown to be pivotal to restrict tissue swelling and osmotic cell death. However, EPs have to be rinsed thoroughly to maintain permeability and the vitally important nutrient diffusion. IVD/EP specimens can be cultured without losing cell phenotype for up to 2 weeks in the case of bovine tail specimens, 3 weeks in the case of ovine specimens, and 4 weeks or more in the case of rabbit specimens. Cyclic loading is generally superior to no or static loading probably by enhancing fluid flow and cell nutrition, but also direct mechanobiological effects on cells may play a decisive role.

2.5.1 Rabbit model of post-traumatic DD

The development of functional explant models was key to the investigation of post-traumatic DD, because inducing traumatic events in-vivo is ethically not tolerable as well as mechanically and analytically challenging. Alternatively, traumatic DD has been induced either by EP perforation, AF stab incision or compressive overload. The investigation of traumatically induced DD has proven very insightful, because a discrete event triggers DD and therefore facilitates the investigation of the pathogenesis.
Haschtmann et al. presented the first explant model for the investigation of post-traumatic DD, which accounts for the dynamic nature and the high impact energy of burst fractures. Controlled fractures in rabbit IVD/EP specimens were induced with a dropped weight and cultured up to 4 weeks. Catabolic, pro-inflammatory, and pro-apoptotic signaling in the NP was reported, which is in agreement with findings in human degenerated IVDs (see 2.3.2 Degenerative disc disease). The rabbit IVD/EP specimens are well suited for in-vitro experiments: they are small enough to guarantee nutrient diffusion into the IVD but big enough to provide sufficient material for different assays. In addition, the genome of the New Zealand White (NZW) rabbit breed used in the Haschtmann model is largely sequenced, which facilitates gene transcription analysis. Last but not least, it is a widespread and accepted animal in research. For all these reasons, the post-traumatic DD studies presented in this thesis are based on this model.

2.6 References


BACKGROUND


BACKGROUND


Chapter 3

Prior storage conditions and loading rate affect the in vitro fracture response of spinal segments under impact loading


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3.1 Abstract

Traumatic injuries of the spine are mostly the consequence of rapid overload e.g. impact loading. In vitro investigations on this topic usually encompass biomechanical testing using frozen/thawed specimens and employ quasi-static loading conditions. It is generally accepted that a freezing/thawing cycle does not alter mechanical properties for slow loading rates. However, this has never been investigated for high impact velocities. In order to assess the effects of freezing/thawing and the influence of different impact velocities, we loaded 27 fresh and 15 frozen/thawed cadaveric rabbit spinal segments (intervertebral disc with one third of the adjacent vertebrae) with different impact energies and velocities using a custom-made, dropped-weight loading device. Endplate fractures were assessed by micro-CT scans. Specimen dimensions (disc, bone, and total height) and vertebrae bone density (BV/TV) were compared pre- and post-trauma. Energy absorption by spinal segments was quantified by measuring the initial ball rebound. We found that freezing/thawing increased endplate fracture frequency and decreased the energy absorption of the segments. Higher impact velocities increased the energy absorption, while higher impact energy increased both energy absorption and fracture frequency. Two conclusions are drawn: first, under impact loading, freezing alters permanently the biomechanical response, and second, for different impact velocities, different fracture initiation mechanisms apply. Therefore, quasi-static loading of frozen/thawed spinal segments is not a valid model for traumatic endplate injuries. However, caution should be exercised in extrapolating these findings to human vertebrae until tests on larger vertebrae are performed.

Keywords: Storage condition; Freezing; Impact rate; Endplate fracture; Fracture requency

3.2 Introduction

Rapidly acting forces are the cause of most human injuries, including spinal injuries1. Despite the immense clinical significance of spinal injuries, segmental biomechanics have been insufficiently analyzed under dynamic loading2. For the study of vertebral trauma, usually frozen/thawed cadaveric material is subjected to quasi-static loading conditions until failure. However, there are two problems linked to this approach: (i) the effects of a freezing/thawing cycle of spinal specimens, when subsequently subjected to high-rate loading is rarely investigated and (ii) the nature of quasi-static loading conditions differs essentially from high-rate loading3.
A number of studies demonstrated that freezing/thawing cycles did not influence biomechanical testing when quasi-static loading conditions were used\(^4\textendash}^9\), but the effects of high impact velocities remain unclear. In vitro experiments confirm the pivotal role of impact velocities on the characteristics of the resulting spinal injuries. Ochia et al. reported higher dynamic failure loads for human lumbar vertebrae\(^3\). Tests on cadaveric calf lumbar spines revealed that, for the same energy and direction of impact, a high impact loading rate produces fractures with significant encroachment of the spinal canal in contrast to minimal encroachment for fractures at a low loading rate\(^10\). Tran et al. suggest that the extent of bursting of the vertebra depends on the rate of pressurization of the body, which could be related to the rate at which the load is applied. Using a porcine model, failure at low loading rates occurred exclusively in the endplate, whereas failure of the vertebral body appeared with greater frequency at higher load rates\(^11\). Therefore, both tolerance and fracture pattern are strongly dependent on impact velocity.

In this study we investigated for the first time the effects of (i) freezing/thawing of spinal segments under dynamic loads and (ii) different dynamic impact rates. We hypothesize that both parameters, (i) prior storage condition and (ii) loading rate, affect the in vitro fracture response.

### 3.3 Methods

**Harvest of spinal segments**

42 spinal segments from T10/11-L7/S1 (10/animal) were isolated within 12 h after sacrifice from five New Zealand White rabbits (4-5 kg, six months old), as previously described\(^12\). Vertebial bodies were flushed with 0.9 % NaCl containing 55 mM sodium citrate. Specimens were assigned to one of six groups (Table 1). Fresh specimens \((n = 27)\) were cultured in standard media (30 ml DMEM/F12, 5% fetal calf serum (Labforce), 50 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml L-ascorbate) for one week after harvest, frozen specimens \((n = 15)\) were stored at -20 °C for six months and thawed at room temperature prior to testing.

<table>
<thead>
<tr>
<th>Impact</th>
<th>Storage</th>
<th>fresh</th>
<th>frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (low rate – low energy)</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B (low rate – high energy)</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>C (high rate – low energy)</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1. Specimen group distribution.*
Trauma induction

Impact loading was performed using a custom-made, dropped-weight (steel ball) fracture device (Figure 1), which guarantees pure axial load. Three different test conditions were applied (Table 2) under non-sterile conditions. The experiments were filmed (Canon G7; 30 frames per second; 640 x 480 resolution) to determine the energy of initial ball rebound ($E_r$) after impact as percentage of the impact energy. Energy absorption by the specimens was calculated as $1 - E_r$.

![Figure 1. Inducing endplate trauma. a) Rabbit spinal segment and load cell (A) of fracture device. Pistil of load cell covers whole transverse area of specimen b) Custom-made dropped-weight fracture device with load cell (A) with steel ball falling from releasing mechanism (B). The steel ball is held by a magnet. By pressing a lever, the magnet is pulled backwards into a cavity. The reduced magnetic force releases the ball. In a free fall, the ball hits the specimen load cell centrally. This is guaranteed as the base was horizontally aligned prior to testing with a spirit level.](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impact rate [m/s]</td>
<td>1.68</td>
<td>1.98</td>
<td>2.95</td>
</tr>
<tr>
<td>Ball mass [g]</td>
<td>534.32</td>
<td>534.32</td>
<td>172.24</td>
</tr>
<tr>
<td>Drop height [cm]</td>
<td>14.5</td>
<td>20.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Energy level [J]</td>
<td>0.76</td>
<td>1.05</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Table 2. Parameters for impact conditions A, B, and C
PRIOR STORAGE CONDITIONS AND LOADING RATE AFFECT THE IN VITRO FRACTURE RESPONSE OF SPINAL SEGMENTS UNDER IMPACT LOADING

Specimen properties

Specimen dimensions were measured with a vernier caliper (Tesa 00530080; 20 μm accuracy) on the unmounted, unloaded specimens and represent the mean of three measures taken at different positions. The dependent variables measured before and after trauma were: height of specimen (ht), of intervertebral disc (IVD) (hd), and of bone (hb), and bone volume density (BV/TV) of cancellous bone in adjacent non-fractured bone compartments using a micro-computed (μCT) tomography device (μCT 40, SCANCO Medical, Bassersdorf, Switzerland) with a resolution of 12 microns. Additionally, μCT scans (24 micron resolution) of the whole specimen were taken after trauma to assess endplate fractures (Figure 2).

Figure 2. Micro-CT image of a burst spinal segment. a) Sagittal and b) transverse view of a micro-CT reconstructed spinal segment with burst fracture (μCT 40, SCANCO Medical, Bassersdorf, Switzerland).

Statistics

For statistical analysis, the software R (version 2.10.1) was used. All values are indicated as mean value ± standard error of the mean (SEM). The significance level for all test was α = 0.05. For the comparison of specimen characteristics, two-tailed t-tests were performed:

- physical characteristics were compared pre- and post-trauma for each trauma group (fractured and non-fractured) using paired t-test.
- trauma groups were compared for each characteristic using unpaired t-test.
Correlation of the two dependent variables $E_r$ and trauma group was calculated using Pearson's product-moment correlation. For the identification of significant experimental parameters, two statistical models were calculated based on the independent variables storage, impact rate, impact energy, and initial BV/TV.

- model 1: ANOVA test type II on a general linear model of the binomial family for qualitative response (fracture yes/no).
- model 2: ANOVA test type II on a linear model for quantitative dependent variables ($E_r$, difference in ht, hb, hd, initial BV/TV). For reason of graphical representation, in the case of $E_r$, the quantitative BV/TV variable was converted into a qualitative variable with two discrete levels (high and low BV/TV) with the median value as separator.

For both models, no corrections for multiple testing were done\textsuperscript{14,15}. Interactions between the independent values were assessed by including pairwise interaction terms. To avoid model over-fitting, bidirectional stepwise model selection by AIC was performed\textsuperscript{16}. Heteroscedasticity was tested with the Breusch-Pagan test\textsuperscript{17}.

### 3.4 Results

**Validation of the statistical models**

There were no significant interaction terms identified, neither in model 1 nor in model 2. Therefore, interaction terms were dropped from the models for further analysis. Bidirectional stepwise model selection by AIC didn’t reduce model complexity. Breusch-Pagan tests for heteroscedasticity were negative for model 1 and 2.

**Parameters affecting fracture frequency**

An endplate fracture was induced in 10 specimens (24 %) (Figure 2), two with loading condition A, seven with condition B, and one with condition C (Table 2). Three fractures were classified as A2 and seven as A3 fractures, according to the AO classification system\textsuperscript{18}. In 7 of 10 cases, the endplate of the vertebral body with lower bone density broke (data not shown, $p = 0.34$). The endplate fracture frequency was found to be dependent on prior storage condition ($p = 0.05$), initial BV/TV ($p = 0.04$), and impact energy ($p = 0.006$). Freezing/thawing and higher impact energy increased fracture frequency (Figure 3, positive coefficient estimate) whereas higher initial BV/TV decreased fracture frequency (negative coefficient estimate).
Prior storage conditions and loading rate affect the in vitro fracture response of spinal segments under impact loading

Figure 3. Factors affecting fracture frequency. High velocity (2.95 m/s) compared to low velocity (1.68 m/s and 1.98 m/s); Frozen/thawed compared to fresh; initial bone density (initial BV/TV); high energy (1.05 J) compared to low energy (0.76 J). Storage condition, impact velocity and energy are qualitative variables, initial bone density quantitative. Coefficient estimates from model $1 \pm$ SEM. Estimate $> 0$: increased fracture frequency; estimate $< 0$: reduced fracture frequency. Absolute values do not correlate with parameter significance in the statistical model.

Changes in specimen characteristics upon impact loading

Total specimen height decreased upon impact loading, independent of an endplate fracture incidence (with fracture: -4.8%, $p < 0.001$; without fracture: -1.2%, $p = 0.009$) but was significantly greater with endplate fracture (-3.6%, $p = 0.002$) (table 3). In this case, significantly reduced IVD height (-30.5%, $p < 0.001$) accounts for the reduction. In contrast, the reduction of total specimen height in non-fractured spinal segments is an effect of reduced total bone height (-1.0%, $p = 0.26$) which is also reflected in a significant increase in BV/TV (+14.6%, $p = 0.02$). In contrast, in fractured specimens, BV/TV was not significantly increased (+5.0%, $p = 0.26$); in addition, total bone height was increased (+3.1%, $p = 0.08$). The opposite tendencies in bone dimensions for fractured and non-fractured spinal segments resulted in a significant difference between the two groups (4.2%, $p = 0.03$).

For the decrease in disc height, none of the independent variables impact velocity, storage condition, initial bone density, and impact energy could be identified as a significant experimental parameter (data not shown). However, higher initial BV/TV diminishes the reduction of total specimen
height (p = 0.08, data not shown). Furthermore, freezing/thawing enhances compaction of central vertebral bone regions (p = 0.06, data not shown).

<table>
<thead>
<tr>
<th></th>
<th>ht</th>
<th>Hb</th>
<th>hd</th>
<th>BV/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-trauma</td>
<td>12.68 ± 1.04</td>
<td>9.76 ± 0.92</td>
<td>2.92 ± 0.73</td>
<td>0.164 ± 0.032</td>
</tr>
<tr>
<td>post-trauma</td>
<td>12.07 ± 1.03</td>
<td>10.06 ± 0.96</td>
<td>2.01 ± 0.63</td>
<td>0.170 ± 0.034</td>
</tr>
<tr>
<td>change [%]</td>
<td>-4.8***</td>
<td>+3.1 a)</td>
<td>-30.5***</td>
<td>+5.0</td>
</tr>
<tr>
<td>non-fractured</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-trauma</td>
<td>12.66 ± 1.87</td>
<td>10.23 ± 1.34</td>
<td>2.47 ± 0.91</td>
<td>0.173 ± 0.034</td>
</tr>
<tr>
<td>post-trauma</td>
<td>12.50 ± 1.84</td>
<td>10.11 ± 1.37</td>
<td>2.39 ± 0.75</td>
<td>0.191 ± 0.061</td>
</tr>
<tr>
<td>change [%]</td>
<td>-1.2**</td>
<td>-1.0</td>
<td>+18.9</td>
<td>+14.6*</td>
</tr>
</tbody>
</table>

Table 3. Dimensions of spinal segments pre- and post-trauma. Values in mm ± sd. Pre- and post-traumatic values were compared using two-tailed paired t-test. a) p < 0.05 comparing fractured and non-fractured post-trauma. b) p < 0.01 comparing fractured and non-fractured post-trauma.

Energy absorption of spinal segments

The absorbed energy correlates with fracture incidence (correlation coefficient = 0.55, p < 0.001). The percentage of absorbed impact energy decreased when specimens were frozen/thawed prior to testing (-3.4 %, p = 0.05) and increased with higher impact velocity (+4.5 %, p = 0.02) and energy (+5.4 %, p = 0.01) (Figure 4).

Figure 4. Parameters affecting energy absorption by spinal segments in impact loading. Data are represented as difference to the corresponding opposite qualitative value (low impact velocity, fresh specimen, low BV/TV, or low impact energy) and are depicted as percent of applied impact energy. Coefficient estimates from model 2 ± SEM.
3.5 Discussion

For practical reasons, spinal injuries are generally investigated on frozen/thawed specimens under quasi-static loading conditions. Therefore, we investigated the effects of (i) freezing/thawing and (ii) impact loading of spinal segments using different impact velocities.

Effects of freezing/thawing

Many studies report no effects of freezing/thawing on biomechanical properties of bone and spinal segments in particular. However, they did not employ high-impact loading. Here, we show that freezing/thawing matters, under high rate loading. We measured increased fracture frequency, although energy absorption was reduced. Thus, failure load is reduced. This implies weakening of the bone and endplate structure due to freezing/thawing. We speculate that freezing, as suggested by Bass et al., cause micro-fractures to the endplates. The high water content of the NP causes a stronger thermal expansion than the AF when frozen. This bulges the endplate and introduces micro-fractures similar to nuclear over-pressurization in compressive loading. This theory is supported by the increased vertebral bone density of frozen/thawed spinal segments when no endplate fracture occurred (data not shown, \( p = 0.06 \)). Micro-damage to the vertebral bone may enhance bone compaction when impact loaded. However, we were not able to directly verify endplate or bone micro-damage by \( \mu \)CT imaging, due to insufficient imaging resolution.

Frozen/thawed spinal segments absorbed less energy. Therefore freezing can actually be protective against endplate failure. It was reported that freezing/thawing reduces viscous properties of soft tissues and increases vertebral body strength as a consequence of reduced disc hydration. Both effects cause the spinal segment to reflect more and absorb less energy. However, these two potentially protective mechanisms were inferior to the weakening effect of presumed micro-fracturing. Therefore, freezing/thawing increased fracture frequency.

The vertebral aspect ratio and bone micro-architecture of the rabbit spinal segment differ somewhat from that of the human segment. It remains to be demonstrated, whether frozen storage affects human vertebrae and discs similarly. However, it was shown that freezing affects the compressive creep behavior of the similarly-sized porcine IVD.
Effects of different impact velocities

We found that higher impact velocities increased the energy absorption, while higher impact energy increased both energy absorption and fracture frequency. This asymmetry reveals an altered loading response i.e. different fracture mechanisms must apply. Higher energy absorption is strongly correlated with a higher risk for endplate fractures. It is the IVD and endplate that absorb most energy, as bone dimensions and densities did not change substantially. This is in agreement with the proposed mechanism for endplate fracture initiation: increased loading energy increases the intradiscal pressure (IDP), which causes the endplates to bulge into the vertebral body and finally to crack, thereby forcing nucleus material into the vertebral body at a greater rate than the contents of the body can be expelled. This leads to a pressurization and burst of the vertebral body.\textsuperscript{1,10,21}

Since in our study a higher impact rate caused higher energy absorption, but not higher fracture frequency, another mechanism distinct from raised IDP must explain this asymmetry. Finite element modeling has indicated that IDP is independent from loading rate, and for shorter impact durations higher stresses in the outer endplates and vertebral body were observed.\textsuperscript{3,11,25} These areas have a higher failure load.\textsuperscript{3,26} Therefore, higher energy absorption does not lead to a higher fracture frequency. Here, we could corroborate in vitro the proposed dependency of the fracture mechanism on impact velocity from finite-element models.

In 7/10 cases the endplate of the vertebral body with lower bone density broke. In this study this was not significant due to low replicate number (n = 10), but similar experiments on human cadaveric motion segments resulted in the same finding.\textsuperscript{27} In addition, they concluded that vertebral fractures usually affect the cranial endplate, because it is thinner and supported by less-dense trabecular bone, an observation also supported by our own past work.\textsuperscript{24} We could also show that a low initial BV/TV predisposes for an endplate fracture. This is in accordance with earlier findings.\textsuperscript{3} In contrast, a high initial BV/TV prevents loss of spinal segment height. In the case of an endplate fracture, this reduction is caused by disc narrowing and, in non-fractured spinal segments, by bone compression.

The increase in total bone height of fractured spinal segments is in agreement with the mechanism which leads to the burst fracture pattern. In this case nucleus material is expelled into the vertebral body causing increased pressure and subsequent burst of the vertebra.\textsuperscript{1} As our loading device only loads spinal segments minimally after the short impact load, axial displacement of vertebral body fragments is not inhibited and may account for a larger total bone height.
In summary, this study demonstrates that (i) freezing/thawing increases the probability for an endplate fracture under impact loading. In addition, (ii) higher impact rate and energy caused higher energy absorption by spinal segments but only higher energy increased fracture frequency. Therefore, different fracture initiation mechanisms apply for different loading velocities. However, the small sample size may limit the interpretation of results. In addition, caution should be exercised in extrapolating these findings to human vertebrae until tests on larger vertebrae are performed.

In conclusion, for the investigation of traumatic endplate injuries, (i) fresh cadaveric tissue should be used and (ii) subjected to high rate loading.

3.6 Acknowledgment

We thank Ladina Ettinger for excellent technical assistance. This study was funded by the Swiss National Science Foundation #310000-122105.

3.7 References


Chapter 4

Fracture of the vertebral endplates but not equienergetic impact load promotes disc degeneration in vitro


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4.1 Abstract

Vertebral endplate damage is associated with intervertebral disc degeneration (DD) in vivo. This finding is confirmed by in vitro investigations. The aim of this study was to further characterize the process of DD using an in vitro full-organ culture model and to elucidate whether significant endplate damage or impact loading alone is pivotal for the initiation of DD.

Rabbit spinal segments (n = 80) were harvested, subjected to pure axial impact loading (n = 40) using a custom-made device and cultured for 28 days. The applied threshold energy (0.76 J) induced endplate fractures in 21 specimens (group A), 19 remained intact (group B). Markers for DD (cell viability, apoptosis, necrosis, matrix remodeling and inflammation) were monitored for 28 days post-trauma in the annulus fibrosus and nucleus pulposus and compared to non-impacted control discs.

Cell viability in group A and B stayed on control level. Group A compared to group B showed enhanced LDH and caspase-3/7 activity, reduced glycosamino-glycan content, reduced aggrecan mRNA, but elevated mRNA for collagen-2, catabolic enzymes (MMP-1/-3/-13), pro-inflammatory (TNF-α, IL-6, IL-8, MCP-1) and pro-apoptotic (Fas ligand, Caspase-3) proteins. Group B compared to control only showed small changes in mRNA levels.

In conclusion, this study demonstrates that burst endplates but not equienergetic loading promotes DD.

Keywords: disc degeneration; burst fracture; impact loading; equienergetic; full organ model

4.2 Introduction

Degeneration of the intervertebral disc (IVD) is of great socioeconomic importance, as it is a principal factor for chronic low back pain and impairment of spinal function1-3. The degenerative process is associated with an aberrant cell-mediated response to structural failures such as endplate fractures, radial fissures and herniations4. Genetic factors, environmental conditions and heavy lifetime occupational load predispose to structural organ failure5, 6. This can occur already with the normal ageing process or it may be induced by excessive mechanical loading, as demonstrated by the application of various combinations of axial loading, bending and torsion7-10. Numerous animal models confirm that disruption of the disc structure initiates a cascade of cell-mediated responses leading to further disruption and progression of degeneration4, 11. Even static loading for several weeks, and to a lesser extent dynamic loading, without structural impairment promote disc degeneration
Fracture of the vertebral endplates but not equienergetic impact load promotes disc degeneration in vitro

Similarly, there is evidence that endplate fractures induce DD\textsuperscript{19-20}. Nevertheless, the question whether a single impact without structural impairment of the endplate, which represents a frequent trauma during a lifetime, is sufficient to promote DD was never addressed. This can be ascribed to the lack of an appropriate model. Chiba et al. embedded rabbit IVD without endplates into alginate to study disc metabolism\textsuperscript{21}. However, the characterization of DD following high impact trauma was not possible until Haschtmann et al. developed an in vitro full-organ model, using rabbit IVD with adjacent endplates\textsuperscript{22}.

Therefore, we extended this model with employment of the adjacent vertebral bone to account for a more physiological situation. We further characterized post-traumatic DD following endplate and vertebral fracture and addressed our specific research question, whether a single equienergetic impact load without vertebral fracture is sufficient to promote DD.

4.3 Methods

Chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) if not stated otherwise.

Full-organ intervertebral disc culture model and trauma induction

80 spinal segments from T10/11 - L7/S1 (10/animal) were isolated within 12 h after sacrifice from eight New Zealand White rabbits (4-5 kg, six months old), as previously described\textsuperscript{22}. Spine segments were carefully dissected, preserving one third (ca. 5 mm) of the adjacent vertebral bodies. Vertebral bodies were flushed with 0.9 % NaCl containing 55 mM sodium citrate. Specimens were cultured for five weeks in 50 ml tubes with standard media (30 ml DMEM/F12, 5 % fetal calf serum (Labforce), 50 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml L-ascorbate). Trauma induction was performed six days post-harvest in half of the specimens, using a custom-made dropped-weight (172.3 g steel ball, 0.76 J) fracture device (Fig. 1), which guarantees axial load. Pilot-experiments revealed 0.76 J as the threshold energy for endplate failure, at which the endplate was expected to fracture in 50 % of the specimens. The initial ball rebound is a reliable indicator for an endplate fracture. A strong ball rebound indicates no endplate fracture (group B), whereas no or very little rebound indicates substantial energy absorption and endplate fracture (group A). Conclusive group assignment was done when dissecting specimens for the assays. The visual inspection was consistent with the classification by initial ball rebound. Discs from the same vertebral level were assigned to each assay for all sampling points (day 1, 3 or 7, 14, and 28...
after trauma) (Table 1). The vertebral levels of the control groups were adjacent to the test group levels for all assays.

<table>
<thead>
<tr>
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<th>day 14</th>
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<td>2</td>
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<td>Histology</td>
<td>1</td>
<td>1</td>
<td>2</td>
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</tbody>
</table>

Table 1. Specimen group assignment. The assignment of trauma specimens to group A or B depended on the type of fracture introduced. a For methodological reasons, cell viability of all NP specimens at day 1 could not be assessed. b Day 7 for Live-Dead and GAG/DNA.

Figure 1. Inducing endplate trauma. a: Rabbit spinal segment and load cell (A) of fracture device. b: Custom-made dropped-weight fracture device with load cell (A) and steel ball falling from releasing mechanism (B).
Quantitative real-time PCR (qPCR)

Whole specimens were collected in 20 ml RNAlater (n = 3/group/day), agitated for 12 h at 25°C, for two days at 5°C and stored at -20°C until further processing. RNA of nucleus pulposus (NP) and annulus fibrosus (AF) were isolated separately as described previously. 20% (10 µl) of purified RNA was used for cDNA synthesis (iScript cDNA Synthesis, BioRad) and 2.5% of it for qPCR in 20 µl iQ SYBR Green (BioRad) containing 2 µM sense and antisense primer (Table 2). qPCR (IQ5, BioRad) was run with the following settings: denaturation: 95°C (10 s, 1 cycle), 50 amplification cycles: 95°C (5 s), 62°C (40 s); followed by melting curve analysis. Data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as fold change compared to control group. No data indicate no detected mRNA either in control, test or both samples.

<table>
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<th>antisense (5’ to 3’)</th>
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<td>Fasl</td>
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Table 2. Primer sequences for real-time PCR.  a COL1, collagen-1; COL2, collagen-2; AGG, aggrecan; MMP1, metalloproteinase-1; MMP2, metalloproteinase-2; MMP3, metalloproteinase-3; MMP13, metalloproteinase-13; MCP1, monocyte chemotactic protein-1; IL6, interleukin-6; IL8, interleukin-8; TNFa, tumor necrosis factor alpha; Casp3, caspase-3; Fasl, fas ligand

Assessment of cell viability

Cell viability was assessed separately for NP and AF. Digestion of specimens (n = 3/group/day), Live/Dead® assay, and imaging was done as described previously. Cells were counted using image analysis software (ImageJ V1.43m, National Institute of Health, Bethesda, MD, USA).
Quantification of glycosaminoglycan

In order to quantify GAG content (n = 3/day), the Alcian blue binding assay was described previously. Optical density was measured (600 nm) and normalized to disc weight.

Quantification of DNA

DNA content was quantified with bisbenzimidol fluorescent dye (Hoechst 33258). Specimens (n = 3/day) were prepared as for GAG measurements and diluted 1:3000 with 10 mM tris(hydroxymethyl)-aminomethane, 1 mM Na_2EDTA dehydrate, 100 mM NaCl. Calf thymus DNA served as standard. Fluorescence was detected with Hoefer DyNAQuant (Amersham Biosciences, San Francisco, USA) and normalized to disc weight.

Assessment of apoptosis

Apoptosis was assessed by measuring caspase-3/7 activity (Caspase-Glo®, Promega, Mannheim, Germany). Half annular samples (n = 3/group/day) were frozen in liquid N2, pulverized with a mortar and pestle and homogenized with a Polytron mixer in 1.5 ml lysis buffer (25 mM HEPES (pH 7.5), 2 mM EDTA, 5 mM MgCl_2, 5 mM DTT (Invitrogen), 1 mM PMSF, 1 mM benzamidine). Debris was removed by centrifugation. Duplicates of 100 µl of supernatant or lysis buffer were measured according to the manufacturer’s protocol. Specific luminescence was calculated by subtracting read values from lysis buffer and normalizing to protein concentration determined by a Bradford assay. Data are presented as percent of control group of the corresponding day.

Quantification of lactate dehydrogenase (LDH) activity

LDH activity, as an indicator for cell necrosis, was measured in cell-free culture supernatant at day 1, 3, 6, and 8 post trauma with the Cytotoxicity Detection Kit (Roche, Basel, Switzerland). Supernatants (control: n = 17/day; trauma: n = 18/day) were frozen at -80 °C until analysis. Duplicates of 100 µl of sample or standard media (blank value) were processed according to the manufacturer’s protocol and measured with a plate-reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). Data were normalized to control of day 1 and presented in percent.
Histology

After fixation in 4 % buffered p-formaldehyde, specimens were washed, dehydrated and embedded in PMMA. Samples were cut sagittally in 6 µm slices with a microtome (HM360, Microm International AG, Switzerland) and stained with Mason-Goldner trichrome. Images were taken with Leica DMRB microscope with IM50 Leica imaging software and stitched using AutoStitch V2.2 (University of British Colombia).

Statistics

For statistical analysis, the software R (version 2.10.1) was used. Non-parametric signed rank tests (Wilcoxon or Kruskal-Wallis) were performed to detect significance (p-value < 0.05) between groups. For qPCR experiments, the geometric mean was calculated as the average value. A difference of at least one PCR cycle was considered significant. For the correlation analysis, ANOVA on a linear model was performed.

4.4 Results

With the applied trauma-protocol endplate fracture occurred in 21 specimens (group A; Fig. 2). In 19 specimens (group B) no visible or only minor vertebral bone fractures were induced without an endplate fracture. As gene transcription levels in the AF of all genes monitored did not differ from the control group, data presented here refer only to the NP. There were insufficient specimens without endplate fractures to allow mRNA quantification at day 28 in group B.

Figure 2. Micro-CT image of a burst spinal segment. a: Latero-sagittal and (b) transverse view of a micro-CT reconstructed spinal segment with burst fracture (µCT 40, SCANCO Medical, Bassersdorf, Switzerland).
Apoptotic and necrotic cell death

Cell viability. There was no difference in cell viability in the AF for group A and B for all time points analyzed (p = 0.27). Cell viability did not decrease up to 28 days (avg. 92 ± 4%, n = 24) except for the AF on day 28 (-8.5%, n = 3, p = 0.20). NP cell viability did not decrease up to 28 days, neither in control group (87 ± 13%, n = 9, p = 0.66) nor in group B (93 ± 5%, n = 4, p = 0.13). There was no difference between control group and group B (p = 0.35). NP material was very limited in group A specimens due to expulsion of the NP during trauma induction. An individual analysis for group A and B was not possible.

Necrotic cell death. LDH activity in media differs for group A and B (each n = 9/day) (Fig. 3): a significant increase was measured up to three days post trauma in group A (day 1: 227 ± 81%, p < 0.001; day 3: 115 ± 58%, p = 0.05), but not in group B (all time points). Activity decreased significantly for group B (-34%, p = 0.04) and control (-35%, p < 0.001) from day 1 to 3 post trauma.

Figure 3. Burst fracture of the endplate (group A) increased LDH release. Equienergetic impact load without endplate frac- ture (group B) did not have any effect on LDH release.

Apoptotic cell death. Caspase-3/7 activity was elevated in group A up to 28 days (avg. 182%, n = 8, p = 0.05), but not in group B (avg. 102%, n = 4, p = 1.00) (Fig. 4). Activity was higher for all time points in group A but, due to low replicate number, not significant. There was no time dependency of the activity in both groups. Gene transcription analysis corroborates this up-
regulation. Transcription of apoptosis associated genes (Fas ligand, Caspase-3) was enhanced in group A for 7 days post trauma (Fig. 5a). However, Fas ligand mRNA at day 1 was not elevated. Equienergetic impact load (group B) only leads to a small up-regulation the first day post trauma and only for Fas ligand and is followed by a down-regulation two weeks post trauma for both genes. A down-regulation was also measured for group A, although less pronounced and not until four weeks post trauma. However, caspase-3/7 activity and Caspase-3 gene transcription do not correlate (not shown; p = 0.84).

Matrix remodeling

*Anabolism.* GAG/DNA was significantly reduced in group A after ≥7 days (avg. -29 %, n = 4, p = 0.05), but not in group B (avg. +15 %, n = 5, p = 0.35). Most aggrecan and other proteoglycans are bound to collagen-1 and -2, with collagen-2 being the most abundant in the NP. Aggrecan and collagen-1 gene transcription temporarily coincided with the reduced GAG content (Fig. 5b). However, at day 28, collagen-1 was strongly up-regulated in group A. Collagen-2 gene transcription was consistently up-regulated in group A for 28 days, but with decreasing magnitude. In group B, an up-regulation was only measured at day 1. Histological sections (Fig. 6) confirm a change in connective tissue composition in the NP space at day 28 post trauma in group A: the NP stains green. Collapsed annular fibers may also account for this finding.
Figure 5. Nucleus pulposus gene transcription levels in group A and B normalized to GAPDH and control (n(A+B) = 3/day). a) Pro-apoptotic genes: Caspase-3 (Casp3) and Fas ligand (FasL). b) Anabolic matrix genes: collagen-1 (COL1), collagen-2 (COL2), aggrecan (AGG). c) Catabolic matrix genes: Matrix metalloproteinase-1 (MMP1), -3 (MMP3), and -13 (MMP13). d) Pro-inflammatory genes: Tumor necrosis factor-alpha (TNFa), Interlukin-6 (IL6), interleukin-8 (IL8), Monocyte chemotactic protein-1 (MCP1).
FRACTURE OF THE VERTEBRAL ENDPLATES BUT NOT EQUIENERGETIC IMPACT LOAD
PROMOTES DISC DEGENERATION IN VITRO

Catabolism. MMP-1, -3, and -13 show almost identical transcription patterns (Fig. 5c). In group A, an initially strong up-regulation attenuates to control level within four weeks. In group B, a small up-regulation on the first day post trauma reverses to a small down-regulation within three days.

Pro-inflammatory signals. All transcription levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-8, MCP-1) (Fig. 5d) followed a similar time course. In group A, a strong up-regulation the first day post trauma was followed by a return to control level within two to four weeks. 28 days post trauma, a small down-regulation for all genes was measured. The initial up-regulation in group B was milder and already normalized after 3 days. The subsequent down-regulation was stronger than for group A.

Figure 6. Photomicrographs of Goldner trichrome stained sagittal tissue sections 28 days post trauma (original magnification x 50; scale bar length: 0.2 cm). a) Impact loaded specimen with endplate fracture (group A); arrows indicating endplate fractures. b) Impact loaded specimen without endplate fracture (group B). c) Control specimen.
Correlation analysis of gene transcription levels

Monitored genes were grouped into three biological classes (MMPs, pro-inflammatory and pro-apoptotic). Endplate fracture (A) as well as equienergetic impact loading (B) provoked an up-regulation of gene transcriptions in all three classes. These initial up-regulations decayed exponentially within days to weeks to control levels or even below. Therefore, log-transformed data could be well fit with a multiple linear regression model (Fig. 7) ($p < 0.001$, $R^2 = 0.91$). The reduction of the gene over-transcription by time is class dependent. Pro-apoptotic transcription levels decay significantly slower than those of MMPs and pro-inflammatory cytokines ($p < 0.001$) and are trauma group independent ($p = 0.73$). The same was found for the MMPs and pro-inflammatory genes ($p = 0.33$). For all three classes, absolute transcription levels were lower in group B than A (all classes: $p < 0.001$) and group B levels were still significantly different from control group (MMP and pro-inflammatory: $p < 0.001$, pro-apoptotic: $p = 0.002$). Within group A, absolute transcription levels of the three classes were significantly different ($p = 0.01$) but not within group B ($p = 0.78$).

![Figure 7](image_url)  
*Figure 7.* Co-regulation of gene transcription. Log-transformed data were fitted with a multiple linear regression model (pro-inflammatory and pro-apoptotic value from day 1 of group A were not fitted). Group A: filled symbols/continuous lines, group B: empty symbols/dashed lines.
4.5 Discussion

The aim of this study was to characterize post-traumatic DD and to elucidate whether the applied impact energy or the endplate fracture is the pivotal factor to promote DD. Therefore, biological responses after in vitro trauma induction were measured in spinal segments (A) with or (B) without fractured endplates but with equienergetic loading. Biological responses were tracked on protein, polysaccharide and on mRNA level.

On all levels, significant differences between group A and B were measured. Whereas for enzyme activities, group B stayed on control levels, gene transcription of group A and B of all gene classes (anabolic and catabolic matrix remodelling, pro-inflammatory and pro-apoptotic) in the NP differed significantly from control group, with group A showing stronger effects. These findings are not surprising, as regulation of gene transcription is very sensitive, but represents only one prerequisite for the regulation of enzyme activity. However, levels of most genes monitored returned to control level within four weeks. Interestingly, this process can be described with an exponential decay (Fig. 7). Messenger RNA for Fas ligand and IL-8 demonstrated a delayed up-regulation and therefore their data from day 1 were excluded from the regression model. This model accounts for 91% of the variance and reveals an almost identical transcription pattern for pro-inflammatory genes and MMPs within each trauma group, but with a stronger up-regulation for group A than B. These findings clearly support the well-described interplay of MMPs and cytokines and probably reflect the initial features of an attempted repair response to injury. MMPs not only cleave matrix components but also cytokines or their precursors, thereby comprising pro-
and anti-inflammatory effects. On the other hand, pro-inflammatory cytokines (TNF-α, IL1-β) are potent inducers of various MMPs. Therefore, the interplay of MMPs and cytokines is a crucial regulator for the severity of inflammation. TNF-α is a main pro-inflammatory cytokine and one of the first on site. It activates the transcription factor NFκB, which has a myriad of pro-inflammatory cytokines as responsive genes. It was demonstrated that NFκB is a direct modulator of HIF-1α expression, which is an important transcription factor under hypoxia in cartilage (also under normoxia) and is vital to chondrocyte survival. HIF-1α responsive genes include those required for regulation of glycolysis, for survival, apoptosis, autophagy and matrix synthesis (SOX-9, collagen-2, aggrecan). It represents a major upstream link of the regulation of pro-inflammatory and anabolic matrix genes and may explain their concomitant alteration in gene transcription. However, aggrecan synthesis was down-regulated in group A two weeks post trauma, which is
characteristic for degenerated discs. This coincides with the reduced GAG/DNA ratio in group A. Therefore, despite an increased collagen-2 synthesis, renewal of extracellular matrix (ECM) is strongly diminished. Using a rabbit annular laceration model, Anderson et al. also reported an up-regulation of collagen-2 and a down-regulation of decorin. Furthermore, they demonstrated up-regulated collagen-1, fibronectin, MMPs and Fas ligand transcription, which is in agreement with our findings. Yang et al. also stated a loss of GAG content, a down-regulation of aggrecan and an up-regulation of collagen-1 and -2 after puncturing AF mice tails in vivo. In contrast to these non-impact loading models, our impact loading model resulted in down-regulation of collagen-1 during the first two weeks post trauma.

It was shown that trauma induces apoptosis in intervertebral discs in vivo and in vitro. Here, we demonstrated that the endplate fracture is the pivotal factor and that sole equienergetic impact loading is not sufficient for the initiation of this process. Although genes associated with apoptosis (Fas ligand, TNF-α, Caspase-3) were slightly up-regulated in group B, caspase-3/7 activity remained on control level. The up-regulation for these genes was stronger in group A, which resulted in a constantly elevated caspase-3/7 activity. This and the delayed onset of the up-regulation of Fas ligand is in agreement with similar experiments using rabbit IVD/endplate specimens or human thoracolumbar discs. Despite cell death by apoptosis, cell viability only decreased in the AF at day 28 but without a difference between group A and B. Nevertheless, necrotic cell death, measured as LDH activity in the supernatant, was significantly elevated for group A the first three days post trauma but not for group B. As cell viability in the AF stayed at a high level in both groups A and B, the endplate, and not the disc itself, is the primary source of LDH.

The micro-surgical preparation method of the specimens leads to major cell damage in the vertebral bone. Subsequent LDH release can be measured up to 10 days post harvesting. Therefore, LDH activity is still decreasing from day 1 to 3 post trauma in the control group. LDH, together with other cell components is released from necrotic cells. Some of these intracellular components (uric acid, ATP, DNA, HMGB1, HSPs) in addition to some extracellular components (ECM protein fragments, hyaluronic acid, heparin sulfate) possess molecular structures dubbed as DAMPs (damage associated molecular patterns) and are capable of triggering inflammatory reactions by activating DAMP receptors (toll like receptors, inflammasome, RAGE) when released to the extracellular space. Our data supports this theory, as LDH activity correlates well with the increased transcription of MMPs, pro-inflammatory, and pro-apoptotic genes. However, as a self-protective mechanism from exaggerated reactions following minor tissue damage, a
certain damage threshold must be overcome. This prerequisite is not met by impact loading without endplate fracture but very well with fractured endplates. Therefore, the structural impairment of the organ, not the applied trauma energy, seems to be the pivotal factor to initiate DAMP-triggered inflammatory cascades.

The interplay of the processes for inflammation and its resolution is tightly regulated. Pro-inflammatory, pro-apoptotic, catabolic and anabolic processes coexist. It is crucial for the resolution of inflammation that these coexisting processes come to a final halt, otherwise a chronic inflammation may develop leading to a slow and terminal destruction of the organ. In this study, most markers of these processes returned to control levels or below four weeks post trauma except caspase-3/7 activity and collagen-1 gene expression. Therefore, our in vitro model represents a valid method for investigating acute inflammation and degenerative processes in the IVD following vertebral burst fracture. Structural impairment of the vertebral bone without endplate fracture, as partially present in group B, is insufficient to induce DD. Therefore, it is the endplate fracture and its concomitant major structural disruption of the disc that trigger DD. For the investigation of chronic inflammation and proceeding DD, traumatized specimens may be subjected to subsequent concurrent loading or exposure to immune cells.

In summary, endplate fracture, but not equienergetic impact loading, is pivotal for the onset of DD. However, high impact loading without endplate fracture may weaken disc tissue in a structurally sound disc by minimally disrupting native matrix structure and altering gene expression profile (e.g. up-regulation of MMPs). These early degenerative changes predispose for endplate fractures in subsequent high impact loading.

4.6 Acknowledgments

We thank Ladina Ettinger for excellent technical assistance. This study was funded by the Swiss National Science Foundation #310000-122105.

4.7 References


Chapter 5

Severity and pattern of posttraumatic intervertebral disc degeneration depends on the type of injury

5.1 Abstract

Background context. The burst fracture of a vertebra is the result of a complex loading procedure and is often associated with intervertebral disc (IVD) degeneration (DD). Likewise, the presumed etiologies are numerous: (i) the structural perturbation of the IVD/endplate, (ii) the impact loading energy alone, and (iii) the depressurization of the nucleus pulposus.

Purpose. To describe the pathogenesis of posttraumatic DD by comparing the severity and patterns of degeneration from different injury models.

Study design. New data from an in-vitro organ culture study are compared to previous work on the same model system.

Methods. In order to investigate in detail the contribution of each factor (i to iii) to DD, we extended our previous work to compare three different segmental trauma processes in a rabbit full-organ in-vitro model: burst fracture (group A; etiologies (i)-(iii)), equienergetic loading without a fracture (group B; (ii)), and endplate puncturing (group C; (iii)). DD markers (apoptosis, necrosis, matrix remodelling, inflammation) were monitored up to 28 days post-trauma. Gene transcription data were subjected to principle component analysis (PCA) and agglomerative hierarchical clustering (AHC) to identify and compare pathological patterns.

This study was funded by the Swiss National Science foundation; project grant number #310010-122105. All authors have no potential conflict of interest.

Results. Only group A showed the full profile of DD: reduced glycosaminoglycan content, increased caspase-3/7 and lactate dehydrogenase (LDH) activity, elevated mRNA of catabolic (MMP-1/-3/-13) and pro-inflammatory (TNF-α, IL-6, IL-8, MCP-1) proteins. In group B, only catabolic and pro-inflammatory genes were slightly up-regulated. In group C, LDH but not caspase-3/7 activity was increased. Catabolic and pro-inflammatory genes were up-regulated, although less compared to group A. Principle component analysis revealed different transcription patterns for group C.

Conclusions. The structural perturbation of the endplate/IVD, but not the loading energy or nuclear depressurization, promotes DD. In addition, endplate puncturing triggers a different pathogenesis, consistent with a more continuous matrix remodelling process.

Key words. intervertebral disc degeneration, posttraumatic, etiology, burst fracture, impact, depressurization, endplate puncturing, severity, pattern
5.2 Introduction

Burst fractures of the vertebra account for approximately 15% of all major spinal fractures\(^1\),\(^2\). Treatment, if any, focuses on preventing or limiting neurological injury and correcting spinal deformity by stabilizing the vertebra (i.e. spondylodesis, vertebroplasty) and decompressing spinal canal stenosis (e.g. by laminectomy). Despite a possible concurrent degeneration of the involved intervertebral discs (IVD)\(^3\),\(^4\), this is not treated in the primary setting, until it becomes painful in the later course. In this context, tissue engineering approaches (IVD replacements\(^5\), annulus fibrosus AF closure\(^6\)) as well as drug administration (anti-TNF\(\alpha\)\(^7\), anti-IL-1\(^7\),\(^8\), growth factors\(^9\)) are discussed as early interventions. However, their application is primarily focused on degenerative disc disease (DDD)\(^10\) and therefore their utilization in burst fracture scenarios is not established. Regenerative medicine approaches would require multiple methods, as multiple anatomical compartments (endplate, nucleus pulposus (NP), and annulus fibrosus (AF)) are affected\(^11\). Until now, the etiopathology of posttraumatic DD is insufficiently investigated, thus the development of regenerative approaches is only slowly progressing. Until recently, posttraumatic DD investigations were hampered by the lack of an appropriate impact trauma model. Instead DD was alternatively induced either enzymatically by digestion of the NP\(^12\) or mechanically by perforating the endplate\(^13\),\(^14\), by static overloading\(^15\),\(^16\), or by annular stab incision\(^17\). Although these represent valid models for DDD, they lack the dynamic character of impact loading and the concomitant massive structural impairment. In 2006, Haschtmann et al. presented the first in-vitro burst fracture trauma model to study posttraumatic DD\(^18\). In a previous study, exploiting this model, we showed that a single impact load without perturbation of the endplate is not sufficient to promote DD, although the same energy was applied (equienergetic) as for the burst fracture\(^19\). In this study, we further investigate whether nuclear depressurization, which inevitably parallels burst fractures, is sufficient to promote DD without any compression force. Depressurization is induced by endplate puncturing. This trauma is not intended to model any in-vivo situation; it merely serves to isolate one characteristic factor in the potential etiology of post-traumatic DD. Furthermore, we compare in detail the three different pathogeneses (burst fracture, equienergetic loading without endplate fracture, and nuclear depressurization), using principle component analysis (PCA) and cluster analysis to compare transcription patterns of DD marker genes for the different traumas.

We hypothesize that the structural perturbation of the IVD/endplate, which is unique to the burst fracture model, is the major factor causing posttraumatic DD and that different etiologies cause different pathogenesis.
5.3 Methods

This study combines results from previously performed trauma experiments on rabbit spinal segments in our group with new experimental data. Earlier published data from burst fractures (group A) and equienergetic loaded specimens without fractures (group B)\textsuperscript{19} were compared to new data from an endplate perforation trauma model (group C), which was conducted at a later time point. In total data from 99 specimens were compared and statistically evaluated in this study (Table 1). Whereas group A comprises three putative etiologies (structural perturbation of the endplate, loading energy, depressurization of the NP), group B (loading energy) and C (depressurization of the NP) comprise only one each (Table 2). Clearly, no model could be designed which comprises only structural perturbation but not loading energy or nuclear depressurization. Nevertheless, this study design allows determining the contribution of each factor to posttraumatic DD by mutual exclusion.

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Table 1. Specimen group assignment. Experimental setup of groups A and B, see Dudli et al.\textsuperscript{19}. \textsuperscript{1} Day 3 for qPCR and Caspase-3/7 assay

<table>
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<tr>
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Table 2. Three different trauma models facilitate the investigation of three different etiologies of DD. Rows: trauma models; columns: etiologies.

Full-Organ Intervertebral Disc Culture Model and Trauma Induction

IVD/endplate specimens were harvested as previously described\textsuperscript{19,20}. Briefly, specimens from T10/11 to L6/S1 (10/animal) were isolated within 12 h after sacrifice from New Zealand White rabbits (3-4 kg, six months old). Group A and B specimens were dissected as spinal segments (IVD/endplate with approximately 5 mm of the adjacent vertebral bodies). Group C specimens were prepared as IVD/endplate specimens as this allows to better control perforation depth. It was previously verified, that spinal segments and
IVD/endplate specimens respond equally to a burst fracture\textsuperscript{19,20}. Specimens were cultured for 5 weeks in 50 ml tubes with standard media. Trauma was induced 1 week post-harvest. In group A and B, a custom-made dropped-weight (172.3 g steel ball, 0.76 J) fracture device was used, which guarantees a pure axial impact load (Figure 1a). In group C, one endplate of each test specimen was perforated with an 18G needle (Figure 1b). This needle size has been previously shown to induce disc degeneration in in-vivo AF puncturing models\textsuperscript{21}. The needle was drilled through the central part of the endplate and carefully removed without extracting the NP. Care was taken to not compress the disc.

Figure 1. Transverse view of micro-CT reconstructed endplates with (a) a burst fracture (12 \( \mu \)m resolution) and (b) a hole drilled with a 18G needle (24 \( \mu \)m resolution) (\( \mu \)CT 40, SCANCO Medical, Bassersdorf, Switzerland).

Quantifying Markers for Disc Degeneration

Discs from the same vertebral level were assigned to each assay for all sampling points (Table 1). The vertebral levels of the control groups were from the same animal and were adjacent to the test group levels for all assays. All assays were performed as described earlier\textsuperscript{19}, in particular: histology, quantitative real-time PCR (qPCR) of NP cells, quantification of DNA and glycosaminoglycan (GAG) of the whole IVD, lactate dehydrogenase (LDH) activity in culture supernatant as measure for necrosis, and Caspase-3/7 activity in the inner AF as measure for apoptosis. The inner AF was taken as the NP was used for qPCR. The AF has been shown to have increased apoptotic activity in disc degeneration\textsuperscript{19}. 
Statistics

For statistical analysis, the software R (version 2.10.1) was used. Non-parametric signed rank tests (Wilcoxon or Kruskal-Wallis) were performed to detect significance (p-value < 0.05) between groups. qPCR data were evaluated using the $2^{\Delta\Delta C(T)}$ method. A difference of at least 2 cycles was considered relevant.

Gene study

The assessment of expression profiles by the use of principle component analysis (PCA) and clustering algorithms is a key tool in systems biology. It facilitates the identification of co-regulated genes and discrepancies/similarities in the response to treatment options. However, in orthopedic research it is only sparsely used, as transcriptional responses to treatments are often measured only at one time point and only for a few genes. In this study, we measured transcription of 11 genes at 3 time points after 3 different traumas. We implemented PCA and agglomerative hierarchical clustering (AHC) to identify and compare transcription patterns between trauma groups (gene study 1) and to compare the groups at different time points (gene study 2). For gene study 1, logarithmized, centered, un-scaled qPCR data of each trauma group were subjected to PCA. Euclidean distance matrices were calculated based on the first two principal components (PC). AHC was performed using Wards method. The numbers of clusters was determined based on the dendrogram structure (Figure S-5, Figure S-6). Clusters were assigned to one of 6 identified patterns. Patterns were calculated as the average values of the cluster members and normalized to day 1.

Gene transcription data of each time point were also subjected to PCA and AHC (gene study 2). Dendrograms were calculated based on AHC. The height of each branch linkage is a measure for the discrepancy between the two branches (trauma groups).

5.4 Results

Tissue integrity and composition

Endplate damage was extensive in group A, absent in group B, and minor in group C. A burst fracture, as well as endplate perforation, lead to a nuclear depressurization and to a partial loss of the NP (Figure 2a and c).
Severity and Pattern of Posttraumatic Intervertebral Disc Degeneration Depends on the Type of Injury

Figure 2. Photomicrographs of Goldner trichrome stained sagittal tissue sections 28 days post trauma. Arrows indicate sites of endplate damage. (a) Impact loaded specimen with endplate fracture (group A), arrows indicating endplate fractures. (b) Equienergetic loaded specimen without endplate fracture (group B). (c) Endplate punctured specimen (group C). (d) Control specimen.
Whereas in group A the loss of NP material and tissue integrity was substantial, immediate and caused by the overloading, the loss in group C was only moderate. It was caused by swelling of the NP after trauma and therefore delayed and progressive for the first hours after trauma. The NP matrix in a healthy disc has a high proteoglycan and water content and low collagen content, mainly of type II. In Goldner-Masson stained histological sections it appears colorless, except the pericellular matrix (Figure 2d). In group A, the NP space was replaced by an open collagen matrix, represented by the green color in Goldner-Masson-stained histological sections (Figure 2a). In group C, the NP space persisted partially free of cells and connective tissue (Figure 2c). Group B showed no structural changes following trauma (Figure 2b).

The reduced GAG content in group A (-29 ± 18 %, p = 0.05), but not in B (+15 ± 32 %, p = 0.35) or C (-11 ± 28 %, p = 0.30) corroborates this change in matrix composition (Figure 3).

![GAG/DNA normalized to control](image)

**Figure 3.** Glycosaminoglycan content normalized to DNA content of whole IVD’s after ≥ 7 days. Mean values ± standard error.

### Apoptotic and Necrotic Cell Death

LDH activity, as a marker for necrotic cell death, was similarly increased the first day post trauma in group A (227 ± 81 %, n = 9, p = 0.004) and C (203 ± 75 %, n = 10, p = 0.002) but not in group B (101 ± 47 %, n = 10, p = 0.70) (Figure 4a). Activity in group A persisted at a high level up to day 3 (178 ± 90 %, n = 8, p < 0.05), followed by an attenuation to control level. In group C, activity dropped significantly from day 1 to 2 (-82 %, p = 0.01) but remained significantly increased up to 1 week post trauma (day 2: 121 ± 23 %, n = 10, p < 0.05; day 7: 114 ± 15 %, n = 10, p = 0.02). Apoptotic cell death, measured as Caspase-3/7 activity in the inner AF (Figure 4b), was elevated up to 28 days only in group A (avg. 182 ± 42 %, p = 0.05). Group B (avg. 102 ± 35 %, p = 1) and C (avg. 127 ± 26 %, p = 0.89) stayed near control level.
Figure 4. Cell death caused by different IVD trauma. (a) Necrotic cell death measured as LDH activity in culture supernatant, normalized to control. (b) Apoptotic cell death measured as Caspase-3/7 activity in the AF, normalized to control. Mean values ± standard error.

Gene Transcription in the NP after trauma

Endplate puncturing caused a moderate initial up-regulation of pro-inflammatory (IL-1, -6, -8, MCP-1, TNF-α), catabolic (MMP-1, -3, -13) and anabolic (collagen-1 and -2) genes (Figure 5). The pro-apoptotic fas ligand and caspase-3 as well as aggrecan responded only with a minute up-regulation. Whereas most responses were back to control level within 28 days, the matrix remodeling genes collagen-1, collagen-2, MMP-1, and MMP-3 were still up-regulated. Striking are the peak up-regulation at day 3 of MMP-1, -13, and TNF-α and the steadily increasing up-regulation of IL-6. In contrast, a burst fracture caused an immediate and strong up-regulation of pro-inflammatory (IL-6, IL-8, MCP-1) and catabolic (MMP-1, -3, -13) genes, and a moderate up-regulation of pro-apoptotic genes (fas ligand, caspase-3, TNF-α) and the anabolic collagen-2 (Figure S-5). Equienergetic loading caused only a small up-regulation of the chemokines MCP-1 and IL-8 and a very small up-regulation of collagen-2 (Figure S-5). For the burst fracture and equienergetic loading, all transcription levels were back to control level or even below after four weeks.
Figure 5. Transcription of DD marker genes in the NP at day 1, 3, and 28 after endplate puncturing. Data ± SEM were normalized to GAPDH. Genes analyzed were: collagen 1 (COL 1), collagen 2 (COL 2), aggrecan (AGG), matrix metalloproteinase 1 (MMP 1), -3 (MMP 3), and -13 (MMP 13), interleukin-1 (IL 1), -6 (IL 6), -8 (IL 8), tumor necrosis factor-alpha (TNFa), monocyte chemotactic protein 1 (MCP 1), fas ligand (FasL), caspase 3 (Casp-3).

Gene study

Within each trauma group, the first two principle components explain 99% of the variance in expression (gene study 1). AHC based on these two PC’s clustered genes with similar temporal transcriptions (Figure S-5, Figure 6). In group A, 4 different patterns were identified, in group B, 3, and in group C, 5 (Figure S-5, Figure S-6). Patterns were found either in all groups (patterns 2 and 4), only in group A and B (pattern 3), only in group A and C (pattern 1), or exclusively in group C (patterns 5 and 6) (Figure 6). Patterns 1 and 2 explain the temporal transcription of all anabolic and pro-apoptotic genes, independent of trauma type. Their characteristics are an initial up-regulation, followed by a transient attenuation at day 3 and a partial restoration of the up-regulation at day 28 (Figure 6). Patterns 3 and 4 contain mostly catabolic and pro-inflammatory genes of group A and B, but not C. They are characterized by an initial up-regulation followed by a strong decline.
Figure 6. Principle component analysis (PCA) of gene transcription from DD marker genes. (a) Clustering reveals 4 clusters in group A, 3 in group B, and 5 in group C. Numbers on the left of boxes indicate the transcription pattern identified (1 to 6). (b) Time course of the 6 transcription pattern identified. Data are normalized to day 1 post trauma. Numbers correlate with Figure 6a.
In group C, most catabolic/pro-inflammatory genes follow patterns 5 and 6, which are unique to group C. In contrast to patterns 3 and 4, they show a delayed peak up-regulation. MMP-3 and IL-8 are clustered within pattern 2, although they show an initial up-regulation followed by an attenuation. As logarithmized data were fed into PCA, their decline was flattened and consequently they were clustered with the less strongly up-regulated anabolic/pro-apoptotic genes.

Comparing the three groups at each time point (gene study 2), AHC reveals a very high proximity of the response in groups A and C at day 1 ($d = 0.09$), equally high dissimilarities at day 3 ($d > 1.32$), and a moderate proximity of the response in groups A and B at day 28 ($d = 0.61$) (Figure 7). The first 2 PCs capture 95 %, 92 %, and 91 % of the variances, respectively.

![Figure 7](image)

**Figure 7.** Hierarchical clustering of gene transcription data at (a) day 1, (b) day 3, and (c) day 28 post trauma. Short branches indicate similar response.

### 5.5 Discussion

In order to further investigate the etiology of posttraumatic DD, we have extended our prior investigations with new data to compare markers for DD in the IVD after a burst fracture (group A), after equienergetic loading without a fracture (group B), and after endplate puncturing (group C). We found that only the burst fracture fully promoted DD in all markers monitored. Therefore, it is the structural impairment and not only the loading energy (group B) or the nuclear depressurization (group C) that triggers DD (Table 2). Furthermore, we have completed a detailed statistical analysis of temporal gene expression patterns, in which the identification of unique transcription patterns in group C reveal a different pathogenesis for endplate puncturing.
Only a burst fracture fully promotes disc degeneration

As described previously, a burst fracture causes an immediate and strong response of DD markers in different aspects of cell metabolism (catabolism, anabolism, apoptosis, necrosis, inflammation) and of different macromolecules (mRNA, protein, polysaccharide) whereas an equienergetic impact load only causes a very weak response of some catabolic and pro-inflammatory DD markers and only on mRNA level. At most, this may cause early degenerative changes but no DD. Similarly, endplate perforation does not reduce the GAG/DNA ratio (Figure 3) nor does it increase pro-apoptotic protein activity in the AF (Caspase-3/7) (Figure 4b). This is not surprising, as the AF structure stays almost intact except for some partial inward bulging (Figure 2c). The increase of necrotic cell death at day 1 post trauma, followed by a quick attenuation, is in agreement with the minor structural perturbation of the endplate (Figure 4a). The massive impairment in burst specimens causes a stronger and longer LDH release (Figure 4). In group C, mRNA levels of anabolic, catabolic and pro-inflammatory genes were moderately elevated compared to group A (Figure 5, Figure S-5). The persistent up-regulation of the collagen-1 and -2, paired with the still increased levels of MMP-3 and -13 after 28 days, reveals a progressive remodeling of the matrix. However, the simultaneous anabolic and catabolic processes balance out and lead to neither a significant GAG loss nor to reduced disc hydration (-0.72% per day; p = 0.17; data not shown) within 28 days. In contrast, in group A, catabolism overpowers anabolism and causes matrix resorption (Figure 1a, Figure 3).

Altogether, endplate puncturing depressurized the NP by minimally impairing the endplate. This was not sufficient to fully promote DD in all markers monitored. Therefore, depressurization of the NP and the concomitant partial nuclear disintegration is not the etiology of posttraumatic DD. Instead, it is the structural perturbation of the endplate, unique to the burst fracture model that promotes DD (Table 2). Bioactive compounds released from or synthesized by major structural impairments of the endplate seem to play a decisive role. For that reason, posttraumatic loading of the IVD would be detrimental. Consequently, a repeated injury model could be considered as a valid burst fracture approximation model. However, the absence of an endplate/vertebra burst strongly limits the expulsion of the NP into the vertebral body. The co-localized NP and stromal cells (i.e. bone marrow) are suggested to trigger an immune response and to enhance matrix resorption by means of increased RANKL expression. Modic changes are also proposed to be a sequel of this co-localization. Therefore, we suggest employing a burst fracture for the specific investigation of posttraumatic DD.
Trauma dependent pathologies

Organ fate is not only determined by the initial response to trauma but also by the subsequent pathological cascades. Therefore, comparing the temporal courses of gene transcription helps to understand the etiopathology of posttraumatic DD. This knowledge is of utmost importance when planning regenerative medical interventions.

Clustering of the three trauma groups corroborates the similar gene regulations in groups A and C at day 1 (gene study 2; Figure 7a). However, as described above, the organ fates are different. This is in agreement with different co-clusterings at later time points (Figure 7b and c). In particular, a burst fracture causes an immediate and strong up-regulation of the catabolic and pro-inflammatory genes (patterns 3 and 4). The same genes are also initially up-regulated in endplate puncturing, although less strongly (patterns 2, 4, 5, and 6). Therefore, AHC clusters A and C at day 1. In a burst fracture, this initial strong up-regulation is followed by a rapid normalization within 28 days to control levels (patterns 3 and 4). In contrast, endplate puncturing causes a delayed peak up-regulation or even a progressive up-regulation (pattern 6). Consequently, the Euclidean distances are bigger and group A and C do not cluster anymore. Gene transcription levels in group B always remain low. Therefore, distances to groups A and C are high at day 1 and low to group A at day 28. This highlights the different pathogenesis for nuclear depressurization and impact traumas, independent of a fracture incidence. The uniqueness of patterns 5 and 6 in group C supports these findings. Patterns 5 and 6, together with pattern 4, represent the majority of catabolic and pro-inflammatory genes in group C (Figure 6). In groups A and B, they are mainly associated with patterns 3 and 4.

In contrast to the catabolic and pro-inflammatory genes (patterns 3 to 6), the anabolic and pro-apoptotic genes show no trauma dependent regulation, i.e. they belong throughout to pattern 1 or pattern 2. This indicates that apoptosis and anabolism in the NP is a consistent response of the IVD to different trauma types. However, the contra effect of the anabolic pattern 1 (collagen-2) to pattern 5 (MMP-1 and -13) is unique for group C. This underlines a continuing matrix remodeling process after endplate puncturing, which ultimately leads to a different organ fate.

Intriguingly, the pleiotropic TNF-α and IL-6 also seem to exert diverse effects in impact loading traumas (groups A and B) and endplate puncturing (group C). In impact loading, TNF-α seems to have a pro-apoptotic function, as it co-clusters with fas ligand and/or caspase-3 (patterns 1 and 2). In endplate puncturing, it co-clusters with the catabolic MMPs, indicating a pro-inflammatory function. IL-6 is initially up-regulated and co-regulated with
MCP-1 and IL-8 in group A, clearly underlining a pro-inflammatory function. In contrast, the progressive up-regulation in group C is unique and opposes the time-courses of all catabolic, pro-apoptotic, and pro-inflammatory genes. This suggests IL-6 to be acting anti-inflammatory. Indeed, IL-6 is known to induce synthesis of soluble IL-1 receptors as well as TNF-α receptor antagonists\textsuperscript{28}, which attenuate the positive feedback loop of NF-κB. This explains the very moderate up-regulation of TNF-α in the burst fracture as well as the rapid drop in the endplate puncturing model. In-vitro, IL-6 rescues T-cells from entering apoptosis, and protects cells from fas-mediated cell death\textsuperscript{29}. It inhibits Treg differentiation and induces Th17 development\textsuperscript{30}; a detrimental step in auto-immune diseases like multiple sclerosis and rheumatoid arthritis and also proposed for disc degeneration\textsuperscript{25}. In addition, IL-6 is key to bone resorption\textsuperscript{31} and also proposed to be crucial in matrix resorption in the herniated disc\textsuperscript{25}. The diverse regulation of TNF-α and IL-6 in different trauma types emphasizes the importance of understanding the etiopathology of posttraumatic DD.

In conclusion, we provide evidence that the structural perturbation of the endplate is the key etiological factor for posttraumatic DD and that different etiologies cause different pathogenesis. Therefore, an appropriate model selection is pivotal for planning regenerative medicine interventions. However, the suggested in-vitro burst fracture model lacks the involvement of the vascular and immune systems, which both play an important role in DD and low back pain\textsuperscript{32}, and is a static culture system. Therefore, to emulate a more physiological situation, this model should be extended with post-traumatic loading in a bioreactor system and/or with co-culturing of bone marrow cells. The abstract nature of this in-vitro model must be balanced against the ethical challenge of an in-vivo trauma model. In future studies using this in-vitro model, we would also recommend to track changes on the protein level. Finally, the extremely high water content and proportion of notochordal cells in the rabbit NP impose a major restriction on this model. By using other species, e.g. bovine tail discs, it could be overcome.

5.6 Acknowledgment

We thank Ladina Ettinger for excellent technical assistance. This study was funded by the Swiss National Science Foundation #310000-122105.
5.7 Supplements

Figure S-5. Temporal course of gene transcription levels within each cluster. 4 (group A), 3 (group B), and 5 clusters (group C) were identified by AHC within each trauma group.
Figure S-6. First row: dendrogram plots with gene clusters (red boxes) in group A (a1), B (b1), and C (c1). Second row: 2D plot of principle component 2 (PC2) vs. principle component 1 (PC1) with the color representation of gene clusters.
5.8 References


SEVERITY AND PATTERN OF POSTTRAUMATIC INTERVERTEBRAL DISC DEGENERATION DEPENDS ON THE TYPE OF INJURY


Chapter 6

Early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent disc in vitro

6.1 Abstract

Stable burst fractures are often treated conservatively with early mobilization. However, burst fractures also disrupt the adjacent intervertebral disc and trigger acute degenerative changes. Secondary trauma can cause chronic inflammation and disc degeneration. It’s not known whether early physiological loading represents a secondary trauma.

72 rabbit spinal segments were harvested and burst fractures \((n = 36)\) were induced using a dropped-weight device. From day 1 to 28 post-trauma, all specimens were loaded daily \((-1 \text{ MPa}, 1 \text{ Hz}, 2500 \text{ cycles})\). At day 1, 7, 14, and 28, specimens \((n = 9/\text{group})\) were taken for analysis: histology \((n = 2)\), glycosaminoglycan content \((n = 3)\), and qPCR \((n = 4)\). Posttraumatic loading compared to loading alone caused a 65 % glycosaminoglycan loss by day 28. Catabolic \((\text{MMP-1, -3})\), pro-inflammatory \((\text{IL-1, -6, iNOS})\) and pro-apoptotic \((\text{TNF-\(\alpha\), FasL})\) gene transcription in the nucleus pulposus was strongly enhanced for 7 days and persisted up-regulated up to 28 days. Trends were similar but less pronounced in the annulus fibrosus and correlated with the nucleus pulposus. Our findings demonstrate that early physiological loading after burst fracture triggers persistent degenerative changes in the disc. Therefore, the time of patient remobilization and type of physiotherapy should be evaluated carefully.

**Key words:** Burst fracture, Disc degeneration, Posttraumatic loading, Vicious circle

6.2 Introduction

Burst fractures account for up to 17 % of all major spinal fractures and most often occur at the thoracolumbar level \((\text{T11 to L2})\). Besides pain relief, the surgical treatment of burst fractures aims to correct posture and restore stability. Nevertheless, correction is often lost and kyphosis progresses within the first months after surgery\(^2\)\(^-\)\(^5\), similarly to conservatively treated burst fractures\(^6\)\(^,\)\(^7\). Therefore, the optimal treatment remains a topic of debate\(^2\)\(^,\)\(^3\)\(^,\)\(^8\). Interestingly, the clinical outcome relates less to the deformity\(^3\)\(^,\)\(^9\) than to the severity of injury\(^9\), the loss of disc height\(^5\)\(^,\)\(^7\)\(^,\)\(^11\)\(^,\)\(^12\), and the degree of disc degeneration \((\text{DD})\)\(^5\)\(^,\)\(^9\). The dependency of the clinical outcome on the disc health is not surprising, as DD is strongly associated with low back pain \((\text{LBP})\)\(^14\)\(^,\)\(^15\), in particular in conjunction with endplate disruption\(^16\)\(^,\)\(^17\). DD has been seen clinically on MRI at a 4 year follow-up in the discs adjacent to burst vertebrae\(^18\), but not after 2 years\(^7\)\(^,\)\(^12\), indicating that post-traumatic degeneration may be a process of damage accumulation in the disc, similar to idiopathic DD\(^9\). The basis for these processes are built within the first hours
and days after endplate trauma, where catabolic, pro-inflammatory, and pro-apoptotic signaling are strongest. Additional trauma during that time can cause a persistent inflammation and enhance DD, possibly by transforming discs cells into a more active form. In addition, a disc with degenerative changes is also more sensitive to a pro-inflammatory stimuli. This defines a vicious circle of increased cytokine release and increased responsiveness to these cytokines. However, little is known about the mechanisms driving DD from an acute to a chronic response. Given the natural severity of burst fractures, the biological response of the disc is strong and requires weeks until normalization is achieved. We hypothesize that any incremental damage during this time period kicks off this vicious circle. In particular, we hypothesize that even physiological loading during the first 4 weeks after trauma can cause a persistent deterioration of disc signaling and enhance DD. The response of the intervertebral disc to post-traumatic loading is studied in an established organ culture model.

6.3 Methods

Chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) if not stated otherwise.

Full-Organ Intervertebral Disc Culture Model and Trauma Induction.

72 spinal segments (IVD/endplate with ca. 5 mm of the adjacent vertebral bodies) from T8/9 – L5/6 (9/animal) were isolated from eight New Zealand White (NZW) rabbits, as previously described. Specimens were assigned to a control or trauma group, to a sampling day (day 1, 7, 14, or 28), and to one of the following analysis methods: quantitative real-time polymerase-chain reaction (qPCR) (n = 4/day/group), glycosaminoglycan (GAG) quantification (n = 3/day/group), and histology (n = 2/day/group). Discs from the same vertebral level were assigned to each assay for all sampling points. The vertebral levels of the control groups were adjacent to the test group levels for all assays. Burst fractures were induced six days post-harvest (day 0) in the test group (n = 36), using a custom-made dropped-weight fracture device (534 g steel ball, 2 J), which ensures axial loading. From day 1 to 28, all specimens were cultured at 37°C, 5 % CO2 in standard media.

Post-traumatic dynamic loading

From day 1 to 28 after trauma, all specimens (trauma and control) were subjected to daily simulated physiological loading (2500 cycles, 1 Hz, 0.8-1.7 MPa; Instron E10000, Instron International Switzerland). Similar loading
Regimes have been shown to enhance IVD anabolism\textsuperscript{25}. The custom-made loading device allowed for the simultaneous loading of six specimens (Figure 1). Each specimen was loaded with the same force (42 N). At day 1, 7, 14, and 28, 2 hours after loading, 9 specimens from each group were taken for analysis.

**Histology**
Fixation, embedding, cutting, and staining was performed as described previously\textsuperscript{20}.

**Quantification of whole disc glycosaminoglycan**
Nucleus pulposus (NP) and annulus fibrosus (AF) were not treated separately, for a better comparison with previous results\textsuperscript{20}. GAG was quantified using Alcian blue binding assay as described previously\textsuperscript{26}.

**Gene transcription analysis**
We selected 10 genes as marker for degenerative changes (Table 1)\textsuperscript{20,22}. RNA isolation and purification from NP and AF as well as qPCR was done as described previously\textsuperscript{26}. In the trauma group, any non-fibrous material within the nuclear space was considered as NP. Data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as fold change compared to control group.

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\textsuperscript{a} Primer sequences for quantitative real-time PCR. \textsuperscript{b} GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; COL1: collagen-1; COL2: collagen-2; TGF\textsubscript{b}: transforming growth factor beta 1; MMP1: metalloproteinase-1; MMP3: metalloproteinase-3; MMP13: metalloproteinase-13; IL1: interleukin-1 beta; IL6: interleukin-6; TNFa: tumor necrosis factor alpha; iNOS: inducible nitric oxide synthase; FasL: fas ligand. \textsuperscript{b} Temperature used for annealing/extension.
Nitric Oxide quantification

NO in culture supernatants of all samples assigned to day 28 (n = 18/group) was quantified indirectly by its oxidation products NO$_2^-$/NO$_3^-$ by Griess reaction$^{27}$.

Statistics

For statistical analysis, the software R (version 2.15.1) was used. qPCR data were evaluated using the $2^{\Delta \Delta C(T)}$ method. Non-parametric tests (Wilcoxon, Kruskal-Wallis with Benjamin-Hochberg correction) were performed to detect significant differences between control and trauma and between different days. In the case of a non-significant day dependency, data pooled from all days were subjected to a Wilcoxon test. Correlations were calculated using Pearson’s test. GAG, DNA and NO data were evaluated by parametric tests (t-test and TukeyHSD). For GAG and DNA, a two way ANOVA on a linear model was calculated to detect trends. Significance level for all tests was $\alpha = 0.05$. 
6.4 Results

Proteoglycan content and histological findings

GAG and DNA content in the control group as well as DNA in the trauma group did not change during the test period (not shown; \( p = 0.97, 0.98, 0.45 \)). GAG in the trauma group declined steadily \( (p < 0.01) \) and resulted in a 64% loss of GAG by day 28, respectively 65% normalized to DNA (Figure 1). Histological sections confirmed the remodeling of the matrix (Figure 2). In the control group, the NP matrix was colorless at all days. In the trauma group, at day 1 and 7, the NP space showed a light turquoise stain, indicating an open collagen matrix. At day 14 and 28, a red stain was predominant, representing a dense unmineralized collagen I matrix.

![Graph](image)

Figure 1. DNA content, Glycosaminoglycan (GAG) content, and GAG/DNA ratio of total discs normalized to adjacent control samples. Significant differences to control groups are indicated (mean ± SD; **: \( p < 0.01 \), *: \( p < 0.05 \)).
Early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent disc in vitro.

**Figure 2.** Photomicrographs of Masson-Goldner trichrome stained sagittal tissue sections (original magnification x2.5). Blue: calcified bone, red: uncalcified, dense connective tissue matrix, turquoise: less dense connective tissue.
Gene transcription in the Nucleus Pulposus

Endplate trauma caused an immediate strong up-regulation of catabolic (MMP-1, MMP-3), pro-apoptotic (TNF-α, fas ligand), and pro-inflammatory (IL-1, IL-6, iNOS) gene transcription (Figure 3). Peak up-regulation was highest at day 1 (MMP-1, MMP-3, fas ligand, IL-1) or 7 (TNF-α, IL-6, iNOS). Levels at day 14 and 28 were lower than at day 1 and 7 for all catabolic, pro-apoptotic, and pro-inflammatory genes but still above control. Due to the large biological variance, up-regulations at day 14 and day 28 did not reach statistical significance (p > 0.08). The same pattern was found for the anabolic TGF-β, although on lower levels. Collagen-1 was on average 17.5 ± 5.8-fold increased (p < 0.001) but did not reach significance at specific days. Collagen-2 shared the same pattern with collagen-1 and was significantly up-regulated at day 7.

Figure 3. Gene transcription in the nucleus pulposus of fractured spinal segments compared to non-fractured control group. From left to right, genes are grouped into anabolic (COL1, COL2, TGFβ), catabolic (MMP1, -3), pro-apoptotic (TNFa, FasL), and pro-inflammatory genes (IL1, -6, iNOS). Abbreviations are explained in Table 1. Asterisks above lines indicate significant differences between days and asterisks above parentheses indicate significant trauma-control difference of pooled data (only in the case of non-significance at all days). Mean ± SEM; ***: p < 0.01; *: p < 0.05.

Gene transcription in the Annulus Fibrosus

Gene transcriptions in the AF of all catabolic, pro-apoptotic, and pro-inflammatory genes showed almost identical patterns to the NP, but were on much lower levels (Figure 4). The large biological variance impeded statistical significance. After 14 to 28 days, control levels were reached. TGF-β was on average 1.8 ± 0.3-fold increased (p < 0.05) but did not reach significance at specific days. Collagen-1 and -2 were not significantly above control levels.
EARLY PHYSIOLOGICAL LOADING AFTER ENDPLATE FRACTURE INDUCES PERSISTENT DEGENERATIVE CHANGES IN THE ADJACENT DISC IN VITRO

Figure 4. Gene transcription in the annulus fibrosus of fractured spinal segments compared to non-fractured control group. From left to right, genes are grouped into anabolic (COL1, COL2, TGFb), catabolic (MMP1, -3), pro-apoptotic (TNFa, FasL), and pro-inflammatory genes (IL1, -6, iNOS). Abbreviations are explained in Table 1. Asterisks above parentheses indicate significant trauma-control difference of pooled data (only in the case of non-significance at all days). Mean ± SEM; **: p < 0.01; *: p < 0.05.

Correlation analysis of gene transcription

Pooled gene transcription data from the three classes catabolism, apoptosis, and inflammation correlated in the NP as well as in the AF (Table 2). Each class also showed high degree of correlation between NP and AF. Anabolic gene regulation did not correlate with the other three classes, neither in the NP nor in the AF nor between NP and AF. Mutual correlations of all genes are shown in S-Figure 2.

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<th>Catabolism (NP/AF)</th>
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<th>Apoptosis (NP/AF)</th>
<th>Inflammation (NP/AF)</th>
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<td></td>
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<tr>
<td>Inflammation</td>
<td>0.02/0.00</td>
<td>0.57***/0.68***</td>
<td>0.43***/0.36***</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NP vs. AF</td>
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<td>0.78***</td>
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</table>

Table 2. Correlation of gene transcription between four gene classes. Top three rows: correlation between the gene classes in NP and AF. Bottom row: correlation between NP and AF for each gene class. Pearson’s correlation estimate are indicated with level of significance: *: p < 0.05, **: p < 0.01, ***: p < 0.001. The genes associated with each class are: anabolism (collagen 1, collagen 2, TGF-β1), catabolism (MMP-1, -3), apoptosis (TNF-α, fas ligand), and inflammation (IL-1, -6, iNOS).
Nitric oxide in culture supernatant

NO concentrations in the culture supernatant from traumatized specimens were increased at day 1 and 2 after trauma (Figure 5). Afterwards concentrations were on control levels and not different from before trauma (day 0).

![Figure 5. NO concentration in culture supernatant. Values from trauma group were normalized to control group. Statistical differences to control groups are indicated (mean ± SD; *: p < 0.05).](image)

6.5 Discussion

The aim of this study was to evaluate whether physiological loading after a burst fracture causes persistent changes in disc cell metabolism that lead to DD. Therefore, we tracked the biological response of the disc to physiological loading after a burst fracture.

We found that GAG content decreased by 65% with 4 weeks of post-traumatic loading. This is in agreement with strong matrix remodeling seen on histological sections. The transition in the NP from no stain, to green, to red is an indication not only for collapsed annular fibers but also for an aberrant healing response, where anabolic and catabolic processes occur in parallel. This was corroborated by significantly enhanced anabolic (collagen-1, -2, TGF-β) and catabolic (MMP-1, MMP-3) genes in the NP. Catabolic, pro-inflammatory, and pro-apoptotic gene transcription was strongly enhanced in the NP during the first week and persisted on a lower level up to 4 weeks. These mutually correlate to a high degree in both anatomical compartments,
the NP and the AF, and between NP and AF. Generally, the AF showed similar trends, although on a lower level.

In contrast to our own previous study, where post-traumatic loading was not considered\(^2\), GAG loss was more than doubled; catabolic, pro-inflammatory, and pro-apoptotic signaling were persistent in the NP, and the AF exhibited degenerative changes. Therefore, early physiological loading may transform the acute degenerative changes, induced by the burst fracture, into chronic changes causing DD.

The clinical outcome of burst fractures correlates with the severity of trauma and disc health\(^5,7,10^-13\). Hence, confining trauma severity and disc damage is pivotal. Obviously, primary damage due to the injury cannot be controlled, but secondary trauma and damage due to early post-traumatic loading can be. It has been reported that secondary trauma causes a chronic inflammation and enhances DD\(^2\). In the present study, it has been shown that even modest (physiological) secondary loading induce strong and persistent inflammatory changes and cause a substantial GAG loss. Furthermore, the changes in inflammatory gene regulation correlated with changes of catabolic and pro-apoptotic gene transcription. This may indicate interactions between their transcription regulations. Hence, the change in one class also affects the others. It is known, for instance, that the activity of MMPs is not limited to matrix cleavage; they also modulate the inflammatory response\(^2\). Some MMPs, including MMP-3, were found to activate pro-IL-1β. Conversely, IL-1β and TNF-α, which are considered to play a pivotal role in DD\(^3\), are potent inducers of MMP transcription\(^2\), possibly by stimulating the NF-κB pathway\(^3\). IL-1β also increases the sensitivity of the IVD to fas ligand by increasing fas expression\(^3\) (in this study, fas transcription was also increased on average \(11.6 \pm 3.2\)-fold \((p < 0.01)\), data not shown). Conversely, fas ligand can induce the processing and secretion of IL-1β\(^33,34\). In the presence of all these interactions, it is not surprising that degenerative discs were reported to be more sensitive to pro-inflammatory stimuli\(^34\). Therefore, it is of utmost importance to confine the inflammatory response from the beginning and to break these vicious circles of chronic expression of catabolic, pro-inflammatory, and pro-apoptotic genes.

The inflammatory changes are generally resolved within 7-14 days when no additional stimuli follow the primary insult\(^35\). However, additional damage, including polytrauma or lumbar spine stabilization surgery, causes a stronger and longer increase of pro-inflammatory cytokines\(^35,36\). In an avascular structure with low cellularity, like the IVD, this can evolve to a chronic inflammation, leading to organ degeneration\(^33\). Previous in vitro experiments using the same trauma model have proven that complete rest after a burst
fracture limits the chronic expression of catabolic, pro-apoptotic, and pro-inflammatory genes. In contrast, the present study demonstrates that even physiological loading after trauma is sufficient to aggravate DD. This underlines the importance of carefully evaluating the optimal time for patient re-mobilization and the role and type of physiotherapy, to avoid a chronic degenerative process. Even during bed rest, back muscles cause compressive stress on the IVD. Therefore, physiotherapy with heavy loading should be avoided and replaced by instructive physiotherapy to unload the spine as much as possible for some weeks.

Besides inflammation, apoptosis is another hallmark of post-traumatic DD. In this study, we demonstrated that post-traumatic loading of burst spinal segments causes a persistent alteration in pro-apoptotic gene transcription in the NP; the death signaling molecules TNF-α and fas ligand were strongly up-regulated during the first week after trauma and persisted on a lower afterwards. The sustained up-regulation is in contrast to the normalization reported in the burst fracture model without post-traumatic loading but in good agreement with a rabbit model of annular laceration. This is reasonable, because the in vivo model always comprises post-traumatic loading of the disc.

The up-regulation of the anabolic genes collagen-1 and -2 in the NP also coincide with the rabbit model of annular laceration and may be explained by the enhanced transcription of TGF-β1, a major up-stream regulator of collagens. Another effect of TGF-β1 is the translational and post-translational modification of iNOS. This could be the reason why NO was only increased for the first two days after trauma, although iNOS transcription level was increased for a longer period.

In contrast to burst fracture without post-traumatic loading, we report here that the degenerative changes extend from the NP to the AF. This is consistent with the proposed “endplate-driven DD”, where the inner AF collapses into the decompressed NP under loading. The few cells in the nuclear area are unable to repair the extensive damage of a matrix, which is repeatedly loaded. This causes a “frustrated” healing response, with increased cytokine and MMP levels. Interestingly, we see a normalization of the response in the AF within 14 days after trauma, despite daily loading. This is in contrast to chronic inflammation in the AF caused by triple stab injury in rat tails. We speculate that stress levels in the outer annulus, in response to physiological loading, are not sufficient to provoke a chronic response in the absence of direct annular injury, despite the increased load transfer onto the annulus with nucleus degeneration.
The method of load application in this study caused higher nominal stresses in smaller specimens. Although we did not observe a significant dependency of gene expression data on the anatomical level (S-Figure 3), the trend in stress distribution opposes the physiological trend of increasing load caudally and may have contributed to the already high variance of biological samples. In order to increase statistical power in future experiments, more specimens should be included for gene transcription analysis.

Care must be taken when comparing the findings of this study with other studies, in particular to our own previous study investigating burst fractures without post-traumatic loading\textsuperscript{20}. Control group treatments do not match, furthermore animals are obtained from different breeders and different FCS batches are used in the culture media, which influences the base response of a given group. In order to increase the comparability with other studies, more control groups should be included in future studies, in particular a group without burst fracture and without cyclic loading.

Future experiments may be directed towards the identification of pathways mediating the observed gene response. For instance, the correlation of pro-apoptotic and pro-inflammatory gene transcription could be further investigated by immune-histochemical quantification of members of the MAPK pathway family. Immunohistochemistry should also be used to verify the results on protein level. Cytokine concentrations in culture supernatant four days after trauma were at detection limit of standard immune assays (ELISA) (own not published data). Therefore, ELISA may fail to detect low but chronic secretion of cytokines.

In summary, this in vitro study shows that early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent discs, which are characterized by vicious circles of catabolism, inflammation, and apoptosis. As a result, the disc exhibits a substantial loss of proteoglycan and internal structural disruption, two key indicators of degeneration. These changes are likely to turn chronically in patients.

### 6.6 Acknowledgments

We thank Jochen Walser and Peter Schwilch for their help with the design of the loading device. This study was funded by the Swiss National Science Foundation #310000-122105.
6.7 Supplements

S-Figure 1. Custom-made six-specimen loading station for cyclic loading of intervertebral discs. The IVD (A) is placed into a compression fixture, which is screwed into an aluminum cap (B) that tightly fits on a standard 50 ml centrifuge tube. To maintain a sterile environment, a circular piece of natural rubber (C) is clamped between the two parts of the piston (D) and between the piston guidance (E) and the cap (B). The displacement of the actuator of a electrodynamic testing machine is transmitted via a piston interface (F) and a height adjustable steel spring (G) to a known force on the piston (D). The whole unit is autoclavable. Six units are inserted into a support cage (H) surrounding a water bath (J). Water temperature is controlled by circulation through an external temperature controlled water bath with its sensor in the water bath (J) of the loading device.
**S-Figure 2.** Correlation of gene transcription. Lower triangle: correlation in the NP, upper triangle: correlation in the AF. Correlation coefficient is visualized by colored ellipses: color hue and ellipse orientation represent sign of correlation, color saturation and aspect ratio represent degree of correlation. Superimposed asterisks indicate p-value of correlation. Correlation coefficients in the bottom row indicate correlation of the specific gene between NP and AF (****: \( p < 0.001 \); ****: \( p < 0.01 \); *: \( p < 0.05 \)). Lines separate anabolic, catabolic, pro-apoptotic, and pro-inflammatory genes.
S-Figure 3. As a consequence of the smaller cross-sectional area of thoracic discs, they were loaded with higher nominal pressures ($1.66 \pm 0.59$ MPa) than upper ($1.35 \pm 0.46$ MPa) and lower lumbar discs ($0.81 \pm 0.20$ MPa). Representative cross-sectional areas were derived from 70 endplates of 8 NZW rabbits not included in this study, but of the same breed, age, and gender. Endplates were scanned with a micro-computed ($\mu$CT) tomography device ($\mu$CT 80, SCANCO Medical, Bassersdorf, Switzerland) at a resolution of 20 microns. Cross-sectional areas were measured using ImageJ V1.43m (NIH, Bethesda, MD). To test the hypothesis, if the increasing nominal pressure in smaller specimens correlates with gene transcription, the dependency of the relative gene transcription from the anatomical level was calculated. Data of each group (NP trauma, NP control, AF trauma, AF control) were subjected to ANOVA with the two quantitative parameters day post-trauma and anatomical level. p-values for anatomical level are listed for each gene and test group. No single group showed a significant dependency from the anatomical level.

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6.8 References


Chapter 7

Splenic mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro

Dudli S., Ferguson S.J., Haschtmann D.
Manuscript ready for submission
7.1 Abstract

There is evidence from in-vivo studies that the nucleus pulposus has auto-immunogenic properties. Due to the complexity of in-vivo systems, there is still uncertainty as to which compound triggers the immune response and which cell type mediates it. Therefore, we aimed to elucidate with simple in-vitro models if fractured intervertebral disc (IVD)/endplates, extracellular-matrix (ECM) fragments, and nucleus pulposus (NP) cells attract and activate mononuclear cells (MNCs). In addition, we aimed to validate the reported homing of bone marrow derived mesenchymal stem cells (BMSC) to degenerative IVDs. We quantified cytokines in co-cultures of splenic MNCs with fractured IVD/endplates, ECM fragments, or NP cells. Chemotactic potential of ECM fragments and media from fractured IVD/endplates were measured using a Boyden chamber assay. Homing of fluorescently labeled MNCs and BMSCs to fractured IVD/endplates was observed histologically. We found that ECM fragments attract MNCs and cause an up-regulation of macrophage (IL-1, IL-6, TNF-α) and T-cell derived cytokines (IFN-γ, IL-17). Activation of MNC by NP cells was highly variable. Conditioned media from traumatized IVD/endplates did not attract and activate MNCs. MNCs and BMSC could infiltrate the fracture site in the endplate but not the IVD. Taken together, these results demonstrate that ECM fragments possess immunogenic properties and that the immune response depends on a close co-localization with immune cells, as occurs in many disc pathologies.

Keywords: intervertebral disc, autoimmune, mononuclear cells, cytokine, chemotaxis

7.2 Introduction

Low back pain (LBP) has an extremely high lifetime prevalence of up to 84% and is strongly associated with disc degeneration (DD). Structural disruption of the intervertebral disc (IVD) occurring from injury or fatigue failure was proposed as the precipitating factor for DD. The IVD responds with disc cells apoptosis, with a sterile inflammation, and with abundant expression of catabolic enzymes. The structural disruption also represents a compromise of the immune privilege of the IVD, which occurs by the avascular and alymphatic structure and the expression of fas ligand. Upon disruption, the IVD becomes exposed to the immune system, is recognized as “foreign” and an auto-immune response is triggered. Therefore, autoimmunity is a secondary effect subsequent to structural damage, which is presumed to prolong clinical symptoms. Indeed, autografting the nucleus pulposus (NP) into immune active tissue in animal models causes an abundant expression of cytokines (IL-1β/-4/-17/-23, TNF-α, IFN-γ) and infiltration of
activated B- and T-cell, and macrophages\textsuperscript{16,19,24–26}. This is in agreement with findings in herniated and degenerated discs\textsuperscript{9,12,17,27–32}. In addition, nucleus pulposus cells (NPC) were reported to be able to elicit a primary immune response in macrophages and natural killer (NK) cells\textsuperscript{33}. Disc proteoglycans were described to enhance lymphocyte transformation in vitro\textsuperscript{31} and mice immunized with cartilage proteoglycans showed strong mononuclear cell (MNC) infiltration into the IVD and almost completely resorbed IVDs\textsuperscript{34}. A key role of the immune system was also proposed for symptomatic Schmorl’s nodes\textsuperscript{35,36} and Modic changes\textsuperscript{37}. However, due to the complexity of human disc pathologies and in-vivo models, some important questions remain unanswered. First, which compound of the NP triggers the immune response, second, which are the mediating cell types and cytokines, and third, are these cells actively attracted by structurally damaged IVDs? Therefore, the aim of this study was to elucidate, by using simple in-vitro models, if structural impaired IVDs in general, and ECM fragments and NPC in particular, can attract and activate splenic MNCs. In addition, we aimed to validate the reported homing of BMSC to degenerative IVDs\textsuperscript{38}.

### 7.3 Methods

For this study 7 experiments were conducted at different time points using a total of 17 New Zealand White rabbits (female, 4-5 kg, 6 months old). Each experiment consisted of one assay, except experiment 6 and 7, for which two assays each were performed (Table 1).

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\textsuperscript{a} for the homing experiment control specimens form chemotaxis assay were used.

**Table 1.** Seven experiments (Exp) were performed for this study. Rabbits were numbered consecutively (#1 to 17). Each experiment consisted of 1-2 assays for which a number of intervertebral discs (#IVD) were harvested from the indicated segments (segment). They were either used for trauma experiments as full organs (IVD/endplates) or NP-cells (NPC) were isolated by tissue digestion. Digestion supernatant (ECM) was also used for some experiments. Except for rabbit #10-11, either mononuclear cells (MNC) from 1 or 2 spleens (#sp) or bone-marrow derived stem cells (BMSC) from the femur were isolated. Abbreviations: ELISA : enzyme-linked immunosorbent-assay ; qPCR : quantitative polymerase-chain-reaction. \textsuperscript{a} for the homing experiment control specimens form chemotaxis assay were used.
For all experiments, IVD/endplate specimens were isolated as previously described. Chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) if not stated otherwise. All solutions were prepared using endotoxin free water and supplemented with Polymyxin B, an endotoxin scavenger (except for experiment 6b, and 7b).

Isolation of rabbit mononuclear cells (MNC) from the spleen

The spleen cell population in rodents is about 5-10 % macrophages/monocytes, 35 % T- and B-cells each, 15-20 % Null cells. We used the heterogeneous spleen cell population to study the immunogenic properties of the NP, because it not only allows direct activation of any splenic cell type, but also allows activation that require more than one cell type, i.e. antigen-presentation to naïve T-cells. From the recorded cytokine profile in co-culture experiments, we were then able to deduce the activated cell type. IL-1, -6, and TNF-α indicate macrophage/monocyte activation, IL-4, -17 and IFN-γ T-cell activation.

Spleens were stored for a few hours at 4°C in phosphate buffered saline (PBS) supplemented with 50 mg/ml penicillin and 100 U/ml streptomycin until cells were isolated. In a petri dish, half filled with Hank’s balanced salt solution without Mg2+ and Ca2+ (HBSS), spleens were torn open with two forceps and splenocytes were released using a cell scraper. After disrupting cell aggregates by gentle pipetting, debris was removed by centrifugation (2 min, 50 g). MNCs were isolated from the supernatant by Histopaque-1077 density gradient centrifugation (30 min, 600 g, room temperature). Cells from the buffy coat were washed twice with PBS. Count and viability was calculated by trypan blue exclusion. For experiment 1 and 2, cells were used immediately for co-culture. For experiment 3, 5, and 6 cells were cultured until used at 5 Mio/ml in lymphocyte media (RPMI-1640 HEPES modification, 10 % FCS, 50 μg/ml penicillin, 100 U/ml streptomycin, 10 ug/ml Polymyxin B, 50 μM β-mercaptoethanol, MEM vitamins, MEM non-essential amino acids).

Isolation and culture of NPCs and preparation of ECM mix

NP from harvested IVDs were pooled and digested in a spinner flask for 2 h at 37°C with 4 NPs per ml digestion solution (DMEM/F12, 10 % FCS, 50 mg/ml penicillin, 100 U/ml streptomycin, 0.3 % Pronase (Calbiochem VWR Int., Dietikon, Switzerland), 0.025 % Collagenase P (Roche, Basel, Switzerland), 0.05 % Hyaluronidase). Pooled cells were used immediately for the co-culture experiments 1 and 2. Cell-free digestion supernatant was boiled for 10 min to heat-inactivate digestion enzymes and sterile-filtered. This solution is
Splenically mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro

subsequently referred to as ECM mix. ECM mix was used immediately for co-cultures in experiments 1 & 2, and frozen at -20° C until used in experiment 3.

Co-culture of MNCs with ECM mix and/or NPCs

For experiments 1 and 2, 2 x 10^6 splenic MNCs were co-cultured for 4 days in a 24-well plate in contact with 2 x 10^5 NPCs or ECM mix (equivalent of 2 digested NPs) or both. As a negative control, each component was cultured alone; as a positive control, 25 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 1 mM Ionomycin was added to the triple-culture. In experiment 1, lymphocyte blastogenic transformation (lymphocyte proliferation) was observed at day 4 as an indicator for primary immune response. Culture supernatant was analyzed for TNF-α, IL-1, -2, -17, and IFN-γ by enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s protocols (Cusabio Biotech, Wuhan, China). Data were normalized to cultured control media.

Quantitative polymerase-chain-reaction (qPCR)

For experiment 3, 2 x 10^6 splenic MNCs were co-cultured for 24 h with ECM mix, either 1:1 or 1:3 diluted with lymphocyte media to an equivalent of 1.0 or 0.5 digested NPs per ml. The control group was cultured in 50/50 lymphocyte/standard media (DMEM/F12, 5% fetal calf serum (FCS) (Labforce AG, Münningen, Switzerland), 50 mg/ml penicillin, 100 U/ml streptomycin, 25 mg/ml L-ascorbate) containing the same amount of heat inactivated and sterile filtered digestion enzymes. For the positive control, 25 ng/ml PMA and 1 mM Ionomycin was added. Cultures were run in triplicates. After 24 h, cells were collected by centrifugation (500 g, 10 min, 4° C) and lysed in 0.3 ml peqGold Trifast (Axon lab, Baden, Schweiz). RNA was purified according to manufacturer’s protocol. cDNA synthesis and qPCR were performed as described earlier using the indicated primers (Table 2). Data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as fold change compared to control group.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>sense (5’ to 3’)</th>
<th>antisense (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>aaggccatcaccatccatctcca</td>
<td>ggtgtcgcgtgatgcacactt</td>
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<tr>
<td>TNF-α</td>
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<td>ccgatcaccctgaagtgc</td>
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<td>IFN-γ</td>
<td>ttcttcagcctctctctctctct</td>
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</tbody>
</table>

Table 2. Primer sequences for quantitative real-time PCR. a GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; TNF-α: tumor necrosis factor alpha; IL-1: interleukin-1 beta; IL-6: interleukin-6; IFN-γ: interferon gamma.
Isolation, culture and trauma of rabbit IVD/endplate specimens

For experiments 4 to 7, IVD/endplate specimens were cultured in 20 ml standard media. Burst fractures were induced 6 days post-harvest in half of the specimens (in experiment 7b at day 7 in 5 specimens) using a custom-made dropped-weight (172.3 g steel ball, 1.0 J) fracture device, which guarantees axial load. For experiments 4 and 5, specimens were cultured in 5 ml standard media without FCS supplement after trauma induction. In experiment 5, 6a, and 7a media was collected after 24 h and frozen at -20°C until used for chemotaxis assay. Control specimens from experiment 7a were subsequently used for experiment 7b.

Isolation of rabbit BMSC

Bone marrow was removed from the femurs with a spatula spoon and transferred to HBSS. Aggregates were disrupted by gentle pipetting. After washing twice with HBSS, MNCs were isolated by Histopaque-1077 density gradient centrifugation (30 min, 600 g, room temperature). Cells from theuffy coat were washed twice with PBS resuspended in α-MEM Nucleoside GlutaMax® media supplemented with 15 % FCS, 50 µg/ml penicillin, 100 U/ml streptomycin, 2.5 ng/ml amphotericin B and seeded at 10’000 cells/cm² in plastic culture dishes. After 4 days, non-adherent cells were discarded. Attached cells were considered as BMSCs. Expansion was done in DMEM/F12 supplemented with 10 % FCS, 50 µg/ml penicillin, 100 U/ml streptomycin, and 2.5 ng/ml bFGF (PeproTech). Cells from passage 4 were used.

Co-culture of MNCs/BMNCs with IVD/endplates

Quantification of cytokines in the co-culture system (experiment 4): Trauma (n = 8) and control (n = 8) specimens were cultured in 8 ml standard media in 12-well plates. 8 Mio MNCs/specimen were added to 4 trauma and 4 control specimens. After 24 h media was collected and frozen at -20°C in aliquots of 1 ml until analyzed for TNF-α, IL-1, -6, -17, and IFN-γ by ELISA according to manufacturer’s protocols (Cusabio Biotech, Wuhan, China).

Homing of MNCs (experiment 6b): Cells (10 Mio/ml) were stained with 5 µM Vybrant® CM-Dil in pre-warmed HBSS (3 min at 37°C, 15 min on ice, washed twice with HBSS). 4 traumatized and 4 non-traumatized specimens were co-cultured with 8 Mio MNCs in 8 ml lymphocyte media for 4 days in Teflon bags (Teflon FEP Foil, transparent, Typ 100 A, Angst+Pfister, Schweiz) on a shaking platform at 37°C, 5 % CO₂, 13 rpm. Assuming a minimal migration velocity of 1 µm/min and a maximal migration distance of 7 mm, 4 days of co-culture is sufficient. Afterwards, specimens were fixed in 4 % formalin,
Splenic mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro.

Embedded in PMMA, cut sagittally in 6 μm slices and counter-stained with Prolong® Gold Antifade with DAPI (Invitrogen, Basel, Schweiz). Cell fluorescence was observed on an inverse microscope (Leica DM IL, filters: A BP340–380 nm and N2.1 BP515–560 nm, 10x magnification). High-resolution digital photos were taken with both filters and superimposed using ImageJ v1.46r (NIH, Bethesda, MD).

Homing of BMSCs (experiment 7b): Cells (10 Mio/ml) were stained with 5 μM Vybrant® CM-Dil (Invitrogen, Basel, Schweiz) in pre-warmed HBSS (3 min at 37° C, 15 min on ice, washed twice with HBSS). 1 Mio BMSC in 30 μl standard media were added onto the fracture site (n = 5) or, for control specimens (n = 4), onto the endplate. After 20 min, 2.5 ml standard media was added. At day 2, 4, and 6, cells were replaced by freshly thawed and labeled BMSCs. Media was changed every other day (BMSC were recycled by centrifugation). On day 7, specimens were fixed in 4% buffered paraformaldehyde, embedded in PMMA, cut coronally in 500 μm slices. Fluorescence was observed on an inverse microscope (Olympus IX51; filter: U-MWIG3 BP530-550). High-resolution digital photos were taken at 2x, 4x, and 10x original magnification.

Chemotaxis assay

Chemotaxis was assessed by Boyden chamber assay using ChemoTx® disposable plates (#101-5, Neuro Probe, Gaitherburg, USA). The chemoattractants tested were: undiluted culture supernatants from traumatized and control IVD/endplates (experiments 5, 7a) and ECM mix undiluted and 1:1 diluted (experiment 6a). Sextuplicates of 29 μl chemoattractant or control media were added to the bottom well. 75’000 Calcein-AM (Invitrogen, Basel, Schweiz) labeled MNC or BMSC in a 25 μl drop of control media were added onto the membrane. Control media was of the same type as the chemotactic solution and was cultured for the same period under the same conditions but without IVDs or ECM mix. After 1 h (MNC) or 6 h (BMSC), fluorescence intensity was quantified in the bottom wells as a measure for cell migration (excitation/emission 485/530 nm; Tecan Infinite F200, Männedorf, Switzerland). 10 nM formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was used as positive control for chemoattraction43. Data are expressed as chemotactic index (CI), which is the ratio of the migration towards the chemoattractant over the migration towards control media.

Statistics

Significantly increased cytokine levels in MNC co-culture experiments were detected using one-way ANOVA and Dunnet post-hoc test with MNC as
reference value. ELISA data from culture supernatants of IVD/endplates were analyzed using 2-way ANOVA with addition of MNC and trauma as treatment variables. Synergism was detected as a significant effect in one of the two individual terms plus a significant and positive effect in the interaction term. If significance was detected within the 2-way ANOVA, Tukey’s HSD post-hoc test was performed to investigate differences between groups. Significance in qPCR was detected using a one-sample t-test. Significant chemotactic effects were detected using one-way ANOVA and Dunnet post-hoc test with control media as reference value. A significance level for all tests was defined as $\alpha = 0.05$. If not stated otherwise, data are depicted as mean ± SEM.

7.4 Results

MNC activation in co-culture with NPCs and/or ECM mix

Lymphocyte blast transformations were observed microscopically in all wells containing the ECM mix (Figure 1b, d, g). The addition of Ionomycin/PMA did not further increase blast transformation (Figure 1g vs. d). MNCs alone or co-cultured with NPCs showed no blast transformation and only minor clusters (Figure 1a, c). ECM mix or MNC lowered the proportion of NPCs attaching to the culture dish (Figure 1c, e, f).

IL-4 was not detectable in the culture supernatant (not shown). The amount of TNF-α, IL-1, IFN-γ, and IL-17 in the culture supernatant showed strong variations between the two independent but identical experiments 1 and 2 (Figure 2). Absolute levels of TNF-α and IL-1 were similar in both experiments but did not correlate ($p > 0.43$). IFN-γ and IL-17 concentrations were about 10-times higher in experiment 1 but showed good correlation to experiment 2 (IFN-γ: $p < 0.05$, cor = 0.83; IL-17: $p < 0.001$, cor = 0.96). In experiment 1, levels of TNF-α and IL-1 correlated ($p = 0.06$, cor = 0.45) as well as levels of IFN-γ and IL-17 ($p < 0.001$, cor = 0.98). In experiment 2, all 4 cytokines correlated mutually with each other ($p < 0.01$, cor > 0.53). In experiment 1, the co-culture of MNC with ECM mix or NPCs increased significantly IFN-γ and IL-17 concentrations. The co-culture with both ECM mix and NPCs showed an additive but not synergistic behavior of the cytokine secretion. MNC alone or cultures lacking MNC showed no increase in IFN-γ and IL-17. TNF-α and IL-1 were never significantly increased in experiment 1, except TNF-α in the co-culture of MNC and NPCs. In contrast, in experiment 2, IFN-γ and IL-17 were, besides the PMA/Ionomycin control, only increased in the co-culture with ECM mix. However, IL-17 did not reach significance ($p = 0.11$). TNF-α and IL-1 levels in experiment 2 were increased in the co-cultures of ECM mix with MNCs or
Splenic mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro

NPCs. Interestingly, TNF-α and IL-1 levels were lower in the triple co-culture MNC/NPCs/ECM mix. The co-culture of MNC with NPCs showed no increase in none of the cytokines in experiment 2.

IL-1β and -6 gene transcription was increased in MNCs when co-cultured for 24 h with 1:1 diluted but not with 1:3 diluted ECM mix (Figure 3). TNF-α and IFN-γ gene transcription were not affected by co-culturing with ECM mix (not shown).

Figure 1. Lymphocyte blast transformation seen on microphotographs of different co-culture systems after 4 days (experiment 1). Brightfield images were taken with 10x original magnification. (scale bar = 0.1 mm)
Figure 2. Cytokines in co-culture of MNC with NPCs and/or ECM mix in two independent but identical experiments. Data were normalized to cultured control media. Significant differences to MNCs cultured alone are indicated (*: p < 0.05; **: p < 0.01; ***: p < 0.001).
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Figure 3. IL-1 and 6 gene transcription in MNCs co-cultured with ECM mix at dilutions of 1:3 and 1:1 (equivalent of 0.5 and 1.0 digested NP, respectively), normalized to control. 25 ng/ml PMA and 1 mM Ionomycin served as positive control. Significant differences to control media are indicated (*: p < 0.05; ***: p < 0.001).

MNC activation in co-culture with traumatized IVDs

Significantly higher amounts of the pro-inflammatory cytokines IL-1 (p < 0.001), TNF-α (p < 0.001), and IL-6 (p < 0.001) were found in culture supernatants from trauma specimens when compared to control specimens independent of whether MNC were co-cultured or not (Figure 4).

Figure 4. Cytokines in culture supernatant from traumatized and control specimens with or without co-cultured MNCs. Significant differences are indicated (*: p < 0.05; ***: p < 0.001)
Therefore, these cytokines were of discogenic origin. For IL-6, IFN-γ, and IL-17, concentrations were higher in MNC co-culture probes, independent of whether specimens were traumatized or not. Therefore, these cytokines were of MNC origin. Hence, the IVD and MNCs both secrete IL-6. For IL-17, a negative synergism of MNC and trauma was measured (p < 0.05).

Chemotaxis

The ECM mix showed a concentration dependent chemotactic effect on MNC, with the undiluted mix (equivalent of 4 digested NPs per ml) having a CI of $1.29 \pm 0.14$. 10 nM fMLP was strongly chemotactic (CI = 1.92 ± 0.28) for MNCs. Culture supernatants from traumatized IVD/endplate specimens were not chemotactic, neither for MNCs nor for BMSCs (Figure 5).

Homing

Homing experiments of MNCs and of BMSCs to fractured intervertebral discs resulted in similar findings. MNCs (Figure 6) and BMSCs (Figure 7) were able to invade the fracture side in the endplate. MNCs could not go any deeper than the edge of the endplates. Very few BMSC were able to migrate a few
Splenitic mononuclear cells are attracted and activated by nucleus pulposus matrix fragments but do not home to intervertebral discs in vitro.

tenths of a millimeter into the IVD. However, the vast majority of BMSCs adhered to the outer surface of the endplates (Figure 7, top image), where they were originally applied. These findings were consistent for all 5 trauma specimens. Control specimens co-cultured with MNCs or BMSCs showed no immigrated cells.

**Figure 6.** Homing of splenic MNCs to fractured IVD/endplates. a) schematic drawing indicating region where photomicrographs (b, c) were taken. b, c) Representative superimpositions of red and blue fluorescence photomicrographs of 6 μm sagittal sections. b and c are taken from two different trauma specimens. Red: MNCs labeled with DM-Dil, blue: all cell nuclei, labeled with DAPI; original magnification: 10x.
Figure 2. Homing of BMNC to a fractured IVD/endplate. Fluorescence micrographs of a representative 500 μm coronal thick section. CM-Dil labeled BMSC appear bright red, bone and connective tissue show a light self-fluorescence. Top image shows medial part of IVD/endplate with both endplates having fractures, indicated by arrows (2x original magnification). Bottom images are magnifications of indicated regions from top image (A: 10x, B: 5x original magnification) (scale bar = 0.5 mm)

7.5 Discussion

The main goal of this study was to elucidate whether structurally impaired IVDs in general, and ECM fragments and NPC in particular, can attract and activate splenic MNCs in-vitro. We showed that ECM fragments trigger an immune response in MNCs, characterized by lymphocyte blast transformation, by the secretion of IL-17 and IFN-γ and by the up-regulation of IL-1β and IL-6 gene transcription. Furthermore, we showed that conditioned media from traumatized IVD/endplate specimens is not chemotactic for MNCs but that enzymatically generated ECM fragments are chemotactic at high concentrations. However, homing of MNC to traumatized specimens was limited to the actual fracture site in the endplate. In addition, we could not confirm that BMSCs are attracted by and home to traumatized IVD/endplates, as reported earlier38.
Splenogenic Mononuclear Cells are Attracted and Activated by Nucleus Pulposus Matrix Fragments but do not Home to Intervertebral Discs in Vitro

There is abundant evidence that the NP, like other immune-privileged organs, can produce an inflammatory autoimmune response. However, there is still uncertainty about the immunogens and the cell type mediating the response. We found that co-culturing ECM fragments with splenic MNCs increased IL-1 and -6 but not TNF-α and IFN-γ gene transcription in MNCs after 24 hours (Figure 3). After 4 days, increased IL-1 and TNF-α secretion were measured in experiment 2. IL-1, -6 and TNF-α up-regulation indicate macrophage activation. After 4 days, we also found increased IFN-γ and IL-17 secretion, indicating T-cell activation (Figure 2). Visual assessment of lymphocyte blast transformation (Figure 1) correlated with the relative amounts of IFN-γ and IL-17. This data supports findings of activated B- and T-cell infiltration into autografted NP. The approximately 10-times higher IFN-γ and IL-17 concentrations in experiment 1 compared to experiment 2 and not significantly increased TNF-α and IL-1 levels suggests that the response was dominated by T-cells, probably because their relative proportion to macrophages/monocytes was much higher than in experiment 2. High inter-experiment correlations of IFN-γ and IL-17 and their comparable relative amounts to PMA/Ionomycin stimulated MNCs indicate that the extents of T-cell responses were similar. This leaves the absolute amount of lymphocytes as an explanation for the increased levels. NPC proved to be immunogenic as well, but only in experiment 1, where an increased secretion of IL-17, IFN-γ and TNF-α was measured. Pre-sensitization of the MNCs in-vivo, prior to experiment 1, could account for the variation. Pre-sensitization of lymphocytes to NP was already described in disc herniation patients with sequestered or extruded NPs, compared to protruded NPs. As we used the spleens only from one animal for the experiments 1 and 2, pre-sensitization and different relative proportions of splenic cell types, in particular of naïve T-cells can have a considerable impact. In order to clarify the cellular source of the reported cytokines, we suggest intracellular cytokine staining followed by flow cytometric analysis. The short time of co-culture (4 days), the concomitant secretion of IL-17 and IFN-γ, and non-detectable amounts of IL-4 (data not shown), indicate the activation of innate lymphoid cells, such as γδ T-cells, rather than the activation of the adaptive immune system. γδ T-cells can also be activated directly by IL-1 without any other co-stimulation. This is intriguing, as we showed that NPC secrete IL-1 and TNF-α, as well as a response to co-culture with ECM mix (significance was reached only in experiment 2). Activation of NPC in co-culture with ECM mix was also seen microscopically as a reduced cell attachment and spreading (Figure 1).
The way that the ECM mix induces the described response can be explained by damage-associated-molecular-patterns (DAMPs). DAMPS are a heterogeneous group of intracellular and extracellular compounds, which are released after tissue damage from necrotic cells or generated by ECM fragmentation. High levels of DAMPs are associated with many autoimmune diseases. ECM-derived DAMPs like biglycan, versican and fragments of heparin sulfate and hyaluronic acid bind to toll-like receptors (TLR) 2 and 4 and activate NFκb transcription, which results in IL-1 and -6 synthesis in macrophages and IL-17 synthesis in γδ T-cells. This is in good agreement with our findings of increased IL-1 and -6 gene transcription (Figure 3) and increased IL-17 secretion (Figure 2). NPC were also shown to express TLRs (manuscript submitted), which may explain their IL-1 and TNF-α secretion in co-culture with ECM mix. By using TLR antagonists it could be tested, if ECM mix engages TLR signaling and causes the described cytokine response.

The pro-inflammatory cytokines IL-1, -6 and TNF-α are also secreted as a direct response of the IVD to a burst fracture, without the involvement of MNCs (Figure 4). However, co-cultured MNC did not show increased TNF-α, IL-1, -6, -17 and IFN-γ secretion. Therefore, neither macrophages/monocytes, nor T-cells were activated. We speculate that DAMPs have been retained in the IVD and/or their soluble concentration in the media was below the activation threshold for MNCs. In addition, the amount of IL-1 and -6 (5.3 ± 1.1 and 7.9 ± 1.9 pg/ml) released by traumatized specimens is about 1000 times lower than typical concentrations required for the differentiation of naïve T-cells. Therefore, these concentrations showed a regulatory, rather than an activating effect on co-cultured MNCs. However, it should be considered that local concentrations can reach the activation threshold and that a longer culture time could lead to cytokine accumulation. These findings imply that MNC activation only occurs in close co-localization with high concentrations of cytokines, NPCs and/or ECM fragments.

We showed that ECM mix is chemotactic for MNCs at high concentrations. This is in agreement with reported chemotactic effects of several matrix proteins and their fragments on neutrophils and monocytes. However, we could not measure a chemotactic effect of conditioned media from fracture IVD/endplates on MNCs. Similar to activation of MNC (Figure 2), this implies that chemotaxis only occurs in regions of high concentrations of ECM fragments. Necrotic factors, which are released after burst fracture of the IVD/endplate, have been reported to be chemotactic for regulatory T-cells, macrophages, neutrophils and BMSCs. In addition, conditioned media from induced degenerative IVDs was also reported to be chemotactic for BMSCs. However, we could not verify BMSC chemotaxis in our model.
Furthermore, MNCs and BMSCs co-cultured with fractured IVD/endplates were not able to penetrate deeper into the IVD than the actual fracture site of the endplate (Figure 6 and Figure 7). We see two reasons for the restricted homing. First, the NP was not, or only a minor, source of chemotactic DAMPs and chemokines, when compared to the endplate. This is unlikely, as high amounts of the chemokine MCP-1 were shown to be secreted from the NP using the same trauma model. Second, and more likely, cell migration was sterically hindered by the dense ECM structure. Effective homing may require prior resorption of the ECM by catalytic enzymes. Indeed, MMPs are strongly up-regulated after burst fracture and persistently expressed when specimens are physiologically loaded after the trauma (manuscript submitted). However, the immediate application of MNCs/BMSCs after trauma in this study did not allow the MMP to exert their catabolic effect and to create sterical access for co-cultured cells. Sterical access could also be granted by circumferential AF fissures, which are a common sequela after endplate fracture, due to post-traumatic loading. Therefore, continuous application of MNCs or BMSCs to burst IVD/endplates, in combination with post-traumatic loading, would not only be a promising model to study cell homing to IVDs, but also represent more physiological conditions. The continuously generated ECM fragments could constantly stimulate MNCs and induce chronic inflammatory changes, a hallmark of autoimmune diseases and inherent to DD and other IVD pathologies.

In conclusion, MNC are attracted and activated by ECM fragments but only at high concentrations. Therefore, the immune response depends on a close co-localization of ECM fragments and immune cells as occurs in burst fracture, disc herniation, and Schmorl’s nodes.

7.6 Acknowledgments

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7.7 References


SPLenic MONonuclear CELLS are ATtracted and ACTivated by NUCleus Pulpo-
sus MATRIX Fragments but do NOT home to INterVertebral DISCS in ViTRO


Chapter 8

General discussion
8.1 Relevance

The overall goal of the thesis was to elucidate in-vitro the etiopathology of post-traumatic DD and the implication of the immune system in this process. Burst fracture of the vertebra/endplate account for approximately 17% of all major spinal fractures and are associated with DD. There is no doubt that DD is involved in most cases of LBP, in particular in conjunction with endplate damage. However, little was known about the etiopathology of post-traumatic DD and its relation to “idiopathic” DD, because no genuine model was available until recently. Haschtmann et al. established an explant model with controlled induction of a burst fracture. Using a further development of this model, with preservation of the vertebral marrow space and the possibility to apply mechanical loading, the etiopathology of DD was investigated. Structural damage of the endplate/IVD has been identified as the pivotal factor promoting the degenerative process, but not the loading energy or nuclear depressurization. In addition, the pathobiological process has been shown to depend on the type of trauma. Furthermore, it was demonstrated that early physiological loading aggravates the degenerative process and that NP material co-localized with leukocytes triggers an autoimmune response.

In this thesis, a novel model for the investigation of DD following burst fractures of spinal segments was used. Burst fractures were reproducibly induced in rabbit explants using a custom-made dropped-weight device. The applied high velocity impact trauma has mechanically only been investigated on frozen/thawed spinal segments but never on freshly harvested ones. Although it is generally accepted that freezing/thawing does not alter mechanical properties for slow loading rates, the effect of high loading rates were unknown. Indeed, it was found that freezing/thawing alters permanently the mechanical response of spinal segments under high impact rates. It was also found that, for different impact velocities, different fracture initiation mechanisms apply. Therefore, prior biomechanical data on fracture initiation and progression derived from ex-vivo experiments should be interpreted with caution when applying these findings to experiments involving fresh, viable organ specimens.

It is known that the fracture mechanism of a high velocity impact (burst fracture) causes a more severe trauma, measured as extent of canal encroachment, compared to the low velocity impact mechanism (compression fracture). In addition, more severe traumas were shown to worsen clinical outcomes in a 5-year follow-up. Since patients with neurological deficits were excluded from this study, the findings cannot be explained by nerve impingements due to canal encroachment but rather by segmental instability and by a more severe and painful degeneration of the affected IVD. Severity of
DD and the underlying pathobiological processes indeed depend on the type of injury, as reported in chapter 4 and 5. Above all, structural perturbation of the endplate is the pivotal factor required to promote DD. Therefore, the initial response of the IVD to trauma is sensitive to the type of injury, in particular to the extent of endplate damage. Consequently, a compression fracture and a burst fracture will most probably result in different organ responses. Comparing the pathobiological response of the IVD to compression and burst fracture using our in-vitro model would elucidate the role of the impact velocity on the fate of the IVD.

It becomes more and more evident that there is more than one phenotype of DD. Depending on the initial structural failure, ‘EP-driven’ and ‘AF-driven’ DD are distinguished (see 2.3.2 Degenerative disc disease). The essential difference of the two models is that NP depressurization precedes AF failure in the EP-driven model and vice-versa, NP depressurization follows AF failure in the AF-driven model. In both cases, NP depressurization plays a decisive role, hence NP depressurization was suggested as a triggering factor for DD. However, the model of NP depressurization by EP perforation alone, presented in chapter 5, disproved the hypothesis that NP depressurization without post-traumatic loading is sufficient to promote DD. This caused only moderate acute degenerative changes on the transcriptional level, but did not lead to proteoglycan loss, the hallmark of DD. In contrast, structural damage of the endplate and the IVD was identified as the pivotal etiological factor triggering DD (chapter 4 and 5). In addition, post-traumatic loading was shown to cause secondary structural damage and to aggravate DD (chapter 6). Therefore, it is not depressurization but structural damage that has the decisive influence on the degenerative process. This finding prompts us to re-evaluate results from a pig model, where DD was successfully induced by endplate perforation. We can now conclude that DD has been brought about by the post-traumatic loading, which is inherent to in-vivo models, and not by NP depressurization. Burst fracture and endplate perforation eventually progress on a similar pathway to complete organ failure under post-traumatic loading. In chapter 5, it was demonstrated that these two types of injury differ not only by the severity of the response but also by the time course and the characteristics of their acute response. Whereas burst fractures cause an overly strong pro-inflammatory and catabolic response during the first days, the response to nuclear depressurization without any axial loading is more moderate but also more persistent. It is therefore likely, that the pathogeneses of burst fracture and endplate perforation also differ in-vivo. The substantial differences of the initial degenerative responses underline the importance to investigate specific research questions on a model which mimics the pathology under investigation, i.e. a burst fracture model to study
thoracolumbar burst fractures. Moreover, results from animal models of DD with a clinically irrelevant induction of DD, like enzymatic digestion of the NP or endplate perforation, should be evaluated critically³.

The different pathobiological processes also underline that an appropriate model selection is pivotal for planning regenerative medicine interventions. High levels of pro-inflammatory and catabolic enzymes differ between trauma types and hence could hamper the successful development of a regenerative approach. On the other hand, specific anti-inflammatory or anabolic treatments at the right time could enhance regeneration, or at least attenuate DD. Especially for non-steroidal anti-inflammatory drugs (NSAIDs), the determination of the exact application time point and duration is crucial as inhibition of PGE2 also increases MMP activity and hence resorption of the IVD⁴.

The considerable different responses of the IVD to different types of trauma and to the occurrence of post-traumatic loading suggest that the pathobiological processes in ‘idiopathic’ DD are considerably different and that traumatic models should be used with reservation for the investigation of ‘idiopathic’ DD. General principles of the degenerative process (apoptosis, catabolism, inflammation) are the same but their cause and effect may vary substantially. Since ‘idiopathic’ DD is more consistent with AF-driven DD, AF laceration may be a more appropriate model, potentially mimicking also the natural development and propagation of lesions within the AF.

The model used in chapter 4 and 5 allowed the identification of the etiological factors and the early degenerative mechanisms after burst fracture. However, it is still not a genuine model for post-traumatic DD, because the described response of the IVD is only of short duration, although very strong. In contrast, degenerated IVDs exhibit a chronic expression of pro-inflammatory, apoptotic, and catabolic proteins¹⁵–¹⁸. There are two essential shortcomings when comparing our model to the in-vivo situation. First, after burst fracture the model does not account for continuous post-traumatic loading, which is omnipresent in-vivo due to muscle tension even when lying in bed, and second, the model is devoid of bone marrow and hence does not allow for any kind of interaction with the immune system. Therefore, the hypotheses that post-traumatic loading aggravates post-traumatic DD (chapter 6) and that an autoimmune reaction takes place after burst fracture (chapter 7) were investigated. It was found that early physiological loading triggers persistent degenerative changes in the disc leading to a doubled rate of proteoglycan degradation within four weeks. It also caused a degenerative response in the AF, which does not take place in burst fracture without post-traumatic loading. This is consistent with the mechanism of EP-driven DD,
where the AF starts to collapse into the NP space when loaded after trauma. Consequently, post-traumatic loading even with a magnitude no higher than physiological loads, represents a secondary trauma leading to accumulative damage and the initiation of vicious degenerative cycles. Nevertheless, post-traumatic loading could not preserve the initial excessive degenerative response for longer than 1-2 weeks. Therefore, the time of patient remobilization and the type of physiotherapy after burst fracture should be evaluated carefully. While complete rest is favorable to prevent the IVD from aggravated DD, vertebral bone mass should be maintained by loading. A reasonable compromise could be complete rest for the first 1-2 weeks after trauma, until the strongest degenerative response is over and the risk to enter a chronic degenerative pathway is minimized, followed by light physiotherapy.

The question of whether the IVD, in particular the NP, has auto-immunogenic competence has been investigated for almost half a century. It is generally accepted that the IVD is an immune-privileged organ. This privilege is compromised by disc herniation and endplate fracture, when NP material is co-localized with immune cells from the blood and the bone marrow. However, autoimmunity was almost exclusively investigated in relation to disc herniations, as this IVD material is comparably easily available. From these studies, and analogous animal models, it is known that herniated NPs cause a pro-inflammatory autoimmune reaction, with the release of a myriad of cytokines, infiltration of immune cells, and the deposition of antigen-antibody complexes. However, it is still unknown what the immunogenic compound is and if a similar reaction takes place in the vertebral bone marrow after axial expulsion of the NP. In chapter 7, it was demonstrated that ECM fragments from the NP have chemotactic potential and that they can activate MNCs. However, homing of MNC to the IVD of burst spinal segments could not be shown. DAMPs may evoke the described pro-inflammatory immune response. The concept of DAMP-mediated sterile inflammation was introduced for the first time less than 20 years ago. Since then, extensive research has revealed the association of many autoimmune diseases with high levels of DAMPs. DAMPs are a heterogeneous group of intracellular and extracellular compounds, which are released after tissue damage from necrotic cells or generated by ECM fragmentation. Therefore, the IVD after burst fracture is a putatively prime source of DAMPs, but this has not yet been investigated. The results presented in this thesis provide evidence for this hypothesis and encourage further studies in this direction. The described cytokine profile corroborates the autoimmunogenicity of the NP and suggests the involvement of IL-17 secreting cells. Similar to DAMPs, the first description of IL-17 is fairly recent and accumulating knowledge highlights its association with autoimmune diseases. Should the hypothesis be proven
that the reported pro-inflammatory immune response is mediated by DAMPs originating from the NP ECM, this would have major implications for the understanding and treatment of many IVD and spine pathologies. Besides disc herniation and burst fracture, Modic changes and Schmorl’s nodes are also characterized by local inflammation and co-localization, or at least strongly enhanced communication, of the NP with immune cells (from the vertebral bone marrow)\textsuperscript{34,35}. As a consequence, these pathologies will only be different representations of the same DAMP mediated pathogenetic process. The implication of DAMPs and IL-17 is well recognized for osteoarthritis (OA)\textsuperscript{36,37} where similar mechanisms take place. Therefore, OA research should be followed carefully when investigating inflammation and immunity of the IVD.

8.2 Critical evaluation of the rabbit model of post-traumatic DD

DD should be studied on human IVD material, if possible. However, this material is often of poor quality and whole IVDs and spinal segments are very rarely available, in particular healthy control specimens. Therefore, the study of IVD biology after segmental trauma like a burst fracture is practically impossible on human tissue and requires an appropriate animal model system. However, there are many differences in cell population, tissue composition, IVD and spine anatomy, development, physiology and mechanical properties, between animal species and human. Their characteristics are discussed in recent reviews\textsuperscript{13,38,39}. Generally, models of DD are classified as either experimentally induced or spontaneous, where experimentally induced models are subdivided as mechanical (alteration of the magnitude or distribution of forces on the normal joint) or structural (injury or chemical alteration)\textsuperscript{38}. Spontaneous models include those animals that naturally develop DD. However, they have the major drawback that the reason for the high rate of DD is unknown\textsuperscript{39}. In rabbits, DD is often induced by stabbing the AF with a scalpel or a needle\textsuperscript{13}. Stabbing depth and needle diameter were reported to affect the rate and severity of DD as well as cell population\textsuperscript{12,40,41}.

In the last two decades, different explant models were developed (see 2.5 Models for the investigation of DDD)\textsuperscript{8,42-44}. Their biggest advantage is that DD can be studied on a native organ structure within a more controlled environment than in in-vivo animal models. Furthermore, no animals have to suffer from painful surgical interventions or discogenic pain.Nevertheless, lives have to be sacrificed, similar to animals designated for meat production.

Using a rabbit explant model, the etiopathology of DD after burst fracture was characterised. To the author’s knowledge, this ‘burst fracture model’ is the first DD model with a clinically relevant initiation of the
degenerative process. Static overload, AF stabbing, and chemical resorption of the NP are effective in inducing DD but do not represent clinical occurrences. Mimicking a more clinical situation, Jünger et al. induced DD by means of limited nutrition in cultured ovine explants and reported reduced cell viability of disc cells. However they could not show increased catabolism, which would be expected in progressive DD. Besides the consistent strong signs for DD in our trauma model, rabbit explants allow for long culture periods up to 4 weeks and are therefore more attractive than other species for the investigation of the degenerative processes (see 2.5 Models for the investigation of DDD). Furthermore, a good ratio of ‘specimens per animal’ makes the rabbit model very cost-effective and ethically defendable.

However, there are some peculiarities that are important to consider when working and interpreting data gathered from rabbit models. First of all, the cellular composition of the NP is notochordal until about 6-12 months of age. The animals used in this study also had mainly notochordal cells in a healthy NP as observed by fluorescence microscopy of isolated cells. Notochordal cells are not only associated with a higher proteoglycan content and a higher ECM synthesis rate, they are also suggested to reprogram the metabolism of surrounding cells towards a more notochordal phenotype. Therefore, the reported fast recovery from burst fractures on the transcriptional level might be caused by a mainly notochordal NP and hence is presumably less pronounced in humans. However, 4 weeks after burst fracture hardly any notochordal cells were found in histological sections. Sections from day 1 showed that most notochordal cells were already lost by expulsion under the high impact load. The remaining cells may have differentiated into or have been replaced by a more chondrocytic cell type. This change in cell phenotype has also been described in a rabbit model of AF stabbing.

Another important consideration when conducting mechanobiological animal experiments is the scaling effect. Since small quadrupeds such as rabbits, mice or rats, require smaller muscular and ligamentous forces to stabilize the spine, their spine is probably exposed to much lower forces. However, the lower intradiscal pressure resulting from smaller forces is largely compensated by a smaller IVD diameter. Not only diameter but also IVD height is reduced and results in similar aspect ratios (height/mean diameter), which is about 0.24 for both, rabbit and human. If considering bone mineral density (BMD) as a measure for nominal loads, forces in the rabbit vertebra should be about 25-40 % lower. On the other hand, BV/TV (ratio of bone to total volume) of rabbit vertebrae is about 60-280 % higher than in human. Taken together, lower BMD and higher BV/TV rather indicate different ways of the bone adapting to resist similar forces than similar bone types resisting to different forces. Whether this difference in bone quality also modulates
the degenerative process is not known. The relation of BMD to DD is not completely understood yet. Increased BMD of the vertebral body but not of the posterior vertebral column or the endplates were associated with more severe adjacent DD\textsuperscript{51,52}. A better understanding of the association between vertebral BMD and DD may provide new insights to the etiology leading to non-traumatic DD.

Last but not least, the small size of rabbit IVDs and vertebrae make the rabbit model generally inappropriate for developing or testing surgical procedures and prostheses. Rabbit models are therefore limited to basic research and to pre-clinical studies, but remain an attractive option for the evaluation of biological therapies.

The used explant model allows for a good control of environmental factors, as the composition of the culture media can be largely controlled. However, the composition of FCS, a standard additive for culture media, can vary substantially between batches and influence the base response of a given group. Another factor which can affect the base response is the origin of the NZW rabbits. Since they are typically inbred within the population of a breeder, animals from different breeders are generally more distinct. In order to achieve a high comparability of the results to other studies, control groups should match between studies. Therefore, care must be taken when comparing the findings of the study presented in chapter 6 with other studies, in particular to the studies presented in chapter 4 and 5.

Heredity is the biggest risk factor for DD, explaining up to 75% of the variance in adult population\textsuperscript{53}. Heredity is not taken into account in animal models but should be kept in mind as a major source of data variance. Genetics may be more important in the predisposition for DD than in the degenerative process itself. Anatomical structure and composition of the AF, NP, and the EP are inherited risk factors and affect the time point for the onset of DD. The genetic effects on the degenerative process once DD is triggered (e.g. burst fracture) are not known. It can be expected that animals are subjected to similar genetic predispositions. The NZW model used in this thesis is an inbred breed and hence the genetic variance is most probably lower than in humans. Statistical analysis confirmed in all of the burst fracture studies that there is no dependency of the progression of DD from the individual. Nevertheless, specimens for a specific test group were selected from different animals to further minimize the remaining variance.

The situation was completely different for immunological studies (chapter 7). An unexpectedly high variance between individual animals was encountered, despite the inbred and similar life-time history of the NZW rabbits. The observed variance may be due to the differences in lymphocyte
composition; lymphocyte maturation causes different B- and T-cell epitopes, and presensitization causes different pools of memory cells. It is conceivable that immunological aspects explain a big portion of the variance of the occurrence of post-traumatic DD. The reported inter-animal immunological variance highlighted a field that has been only sparsely investigated so far, but which should be considered highly relevant for the understanding of DD. Therefore, further studies investigating immunological aspects of DD are critical.

It should be kept in mind that, despite strong signs of DD (GAG loss, inflammation, catabolism, apoptosis) in the burst fracture model, these signs cannot be placed equal to the clinical factors (pain, disabilities) that ultimately lead the patient to consult a doctor. Hence, the intensity of DD markers in a model should not be confused with the intensity of clinical symptoms.

8.3 References


Chapter 9

Conclusion and outlook
9.1 Conclusion and outlook

In this thesis, an in-vitro model of post-traumatic DD has been developed and structural damage was identified as the pivotal factor driving the degenerative process. The findings demonstrate that fracture of the endplate has multiple adverse effects; first, it immediately boosts degenerative processes in the IVD, second, it inevitably leads to accumulative damage of the IVD under post-traumatic loading, and third, it facilitates an auto-immune response by co-localizing expelled NP material with immune cells from the bone marrow.

The identification of the etiology and the gathered understanding of the pathobiological processes of DD facilitate the development of treatment approaches targeting the degenerative process, compared to current treatments aiming to reduce clinical symptoms. Obviously, primary damage due to the injury cannot be prevented, but secondary trauma due to post-traumatic loading can be. Therefore, physiotherapy with heavy loading should be avoided and replaced by light physiotherapy to unload the spine as much as possible during the acute response of the IVD. During this phase, the administration of anti-inflammatory drugs may support the return to normal cell metabolism and hence show beneficial effects on the organ fate. This could be tested relatively simply with our model. A strong degenerative response can be consistently triggered by a burst fracture. The progression of this degenerative process could be monitored and modulated with drugs. In another study, it could be investigated, if steroids or other immune suppressive drugs counteract the onset of the immune response. If the immune response is initiated by signaling through TLRs, TLR antagonists may prove effective. For this reason, at first an appropriate model system needs to be developed where an immune response against the NP can be consistently induced. The burst fracture model presented in this thesis is the only DD model which induces DD with a clinically relevant scenario. In addition, it is the only in-vitro model that allows studying the effects of co-localized leukocytes and their immune response in the native disc environment. Therefore, this model provides a promising basis for the establishment of an IVD-immune model system.

Care must be taken when results from different studies using the same explants models are compared. It is imperative that control groups match. Therefore, findings from the study in chapter 6 cannot be directly compared to the findings from the studies presented in chapter 4 and 5. In order to close this gap, a study comparing the different control groups of these studies should be conducted, i.e. physiological loading vs. no loading of non-traumatized spinal segments.
The biological sequelae of endplate fractures are severe (DD\textsuperscript{6–8}, autoimmune response\textsuperscript{9,10}) and a genuine regeneration is impossible, since post-traumatic loading sustains an inflammatory environment and the constant generation of matrix degradation products feeds a pro-inflammatory immune response in leukocytes. An endplate prosthesis could stop these two detrimental processes. By restoring pre-traumatic load distribution, the excessive production of matrix wear products would be stopped and thereby facilitate normal disc metabolism. In addition, it would dampen the autoimmune response, as it would impede the co-localization of IVD material and immune cells. Nevertheless, no method currently exists for the replacement of the vertebral endplate and the development of such a prosthesis would be challenging. Since an endplate prosthesis without disc replacement is anatomically not feasible, as the two structures are strongly interconnected, a plate-like insert between the endplate and the vertebral body may be a better alternative, although a balance between nutrient permeability and immune cell control would be required.

The detailed description of the pathogenesis of DD provided in this thesis also facilitates the creation of novel diagnostic tools. As an ultimate aim an easy blood test is envisioned, which measures several unambiguous markers of DD (inflammatory, apoptotic, immune status) and, based on the results, a tailored treatment can be designed (drugs, surgical intervention).

In conclusion, this thesis provides a better understanding of the post-traumatic degenerative process in the IVD and points out that post-traumatic loading and immunological aspects can aggravate this process. Although the pathogenesis of DD depends on the etiology, ‘idiopathic’ and post-traumatic DD share the same characteristics, namely inflammation, apoptosis, and matrix resorption. Therefore, our burst fracture model and results from this model are of general interest for the further investigation of DD and facilitate the development of treatment approaches not only for post-traumatic DD but also for ‘idiopathic’ DD.

9.2 References


Appendix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>3-Dimensional</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus Fibrosus</td>
</tr>
<tr>
<td>AGG</td>
<td>Aggrecan</td>
</tr>
<tr>
<td>AHC</td>
<td>Agglomerative Hierarchical Clustering</td>
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<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
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<tr>
<td>BMSC</td>
<td>Bone Marrow derived Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone Volume / Total Volume</td>
</tr>
<tr>
<td>Casp</td>
<td>Caspase</td>
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<tr>
<td>CEP</td>
<td>Cartilage Endplate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
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<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>CT</td>
<td>Computet Tomography</td>
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<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
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<tr>
<td>DD</td>
<td>Disc Degeneration</td>
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<tr>
<td>DDD</td>
<td>Degenerative Disc Disease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleid Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EP</td>
<td>Endplate</td>
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<tr>
<td>FasL</td>
<td>Fas Ligand</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-Methionyl-Leucyl-Phenylalanine</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia-Inducible Factor-t-alpha</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-Mobility Group 1</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-Gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>IVD</td>
<td>Intervertebral Disc</td>
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<tr>
<td>LBP</td>
<td>Low Back Pain</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
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<tr>
<td>MEM</td>
<td>Modified Eagle Medium</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MNC</td>
<td>Mononuclear Cell</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<tr>
<td>NC</td>
<td>Notochordal Cell</td>
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<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>NP</td>
<td>Nucleus Pulposus</td>
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<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
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<tr>
<td>NZW</td>
<td>New Zealand White</td>
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<tr>
<td>OA</td>
<td>Ostheoarthritis</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PC</td>
<td>Principle Component</td>
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<tr>
<td>PCA</td>
<td>Principle Component Analysis</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>PGE₂</td>
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<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
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<tr>
<td>PMMA</td>
<td>Poly(Methyl Methacrylate)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear factor Kappa-B Ligand</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SN</td>
<td>Sinus vertebral Nerve</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
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Dissemination

Journal articles

2013  **Dudli, S.**, Ferguson, S.J., Haschtmann, D. Splenic Mononuclear Cells are attracted and activated by Nucleus Pulposus Matrix Fragments but do not home to Intervertebral Discs in-vitro. (to be submitted)

2013  **Dudli, S.**, Haschtmann, D., Ferguson, S.J. Early physiological loading after endplate fracture induces persistent degenerative changes in the adjacent disc in vitro. (submitted to *J Orthop Res*)

2013  **Dudli, S.**, Haschtmann, D., Ferguson, S.J. Severity and pattern of posttraumatic intervertebral disc degeneration depends on the type of injury. (under revision in *Spine J*)


Conferences


2010  **Dudli, S., Haschtmann, D., Ferguson, S.J.** Fracture of the vertebral endplates but not equienergetic impact load promotes disc degeneration in vitro. Podium presentation at the eCM Conference 28-30.6.2010 in Davos, Switzerland.

2010  **Dudli, S., Haschtmann, D., Ferguson S.J.** Prior storage conditions and loading rate affect the in vitro fracture response of spinal segments under impact loading. Poster presented at the ESB Conference 5.-8.7.2010 in Edinburgh, Great Britain.

**Guest lectures**

2013  “An in-vitro model for post-traumatic intervertebral disc degeneration.” Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich.
Acknowledgment

First of all, I would like to acknowledge the Swiss National Science Foundation for funding this project (#310000-122105).

The story of this PhD began a few days before Christmas 2008. My friend Pius and I had to push hard our bicycles this morning to make it to Esquel, the last bigger town in Patagonia before civilization ultimately gives way to the void and the wind. We were riding our bicycles as fast as we could, but strong head winds were slowing us down. I had to make it to Esquel by noon, because I had a job interview via skype with Prof. Ferguson for this PhD position. I was nervous, first, because I did not know if we would make it to this town on time and second, because it has generally not been easy to find a cyber café in these small towns, which had fast enough internet for running skype properly.

Why am I telling this story? It reflects nicely my gratitude and esteem of Stephen. Which employer would be willing to hire a guy for 4 years without having seen him before face-to-face? Which employer would do the job interview at the time the applicant thinks would suit him best, although he is on vacation? It definitely must be a courageous person who exactly knows what he wants. And it definitely has to be a bicycle freak!

The belief and support in me from the very first moment formed my work and our relationship. I loved my job and I learned a lot from him, not only professionally but also socially. He was not only boss, but also mentor and friend. I am deeply impressed how he effectively combines enthusiasm and serenity in a field driven by deadlines into exciting straightforward research. I feel grateful to you, Stephen, for many things, but in particular for having hired this strange cycling guy 4 years ago and for your enduring support since then until this very moment. Thank you!

To finish the bicycle story: luckily, we found a cyber café exactly on time with fast internet and even with web cams. But I negated the possibility for visual contact during the call as this would probably have lead to an immediate, unfavorable end of the talk. I was wearing a shirt that has been bleached by 19 months cycling in the sun. In addition, my 5-day-beard in combination with my curly, uncombed shoulder long hair provided my more the look of a drunken unsociable weirdo than of an ambitious PhD candidate.

I also feel deeply grateful for Daniel Haschtmann, co-applicant of the SNF project. Despite his full schedule he always has taken time for discussing my projects. As a young researcher in the biomedical field, it was inestimable to have an experienced orthopedic surgeon doing some reality checks on my
experiment proposals. Thank you, Daniel, for the constructive discussions! I would also like to thank you for giving me the original inspiration for my PostDoc project.

Special thanks I would like to dedicate to Willy Hofstetter, co-referee of my PhD. Besides fruitful discussions throughout my PhD, I thank you in particular for your willingness and special efforts to perform an accelerated evaluation of this thesis.

Honestly, without Ladina Ettinger this thesis would be only half the size as she taught me all the methods used during my thesis in the wet lab. However, thanking her just for her technical advices and assistance would be as ridiculous as acknowledging Einstein only for his work in the patent office in Bern. Ladina turned every single working day into an amusing day no matter if we were harvesting rabbit IVDs for 16 hours together or if we just had a 15 min coffee break together. Experiencing her lust for life was highly motivating and provided me also some inspiring distant view to my own work. Thanks to her, I am now also (theoretical) expert in gardening and keeping cats!

Furthermore, I would like to acknowledge Urs Rohrer, Alex Bürki, Peter Schwilch and Jochen Walser for the help with designing and constructing the fracture and loading device. I appreciated their pragmatic and effective hands-on approaches. It is unbelievable how Jochen has always found time for supporting me when I needed his help. I never saw him stressed. I guess this is due to his Bavarian genetics. Our shared love for solid food made us cook Raclette, Fondue, liver- and blood wurst for lunch. Unforgettable! Thank you Jochen, my friend, we stay in touch!

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Curriculum vitae

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Apr.09 – Apr.13 PhD candidate at ETH Zürich, Institute for Biomechanics, Switzerland, under the supervision of Prof. Dr. Stephen Ferguson. Thesis title: "Post-traumatic Intervertebral Disc Degeneration – An In Vitro Study on the Etiopathogenesis"

Jun.07 – Feb.09 Bicycle trip from Anchorage to Fireland. www.panamerica.ch

Apr.07 – May.07 Research fellow at ETH Zürich, Switzerland, Departement of Biosystems Science and Engineering, Group of Prof. Dr. Jörg Stelling

Oct.05 – Mar.07 Master Student at University of Zürich, Switzerland. Minor field of study: Bioinformatics. Master thesis conducted in the Department of Biochemistry under supervision of Prof. Dr. Andreas Plückthun. Thesis title: “Characterization and directed evolution of a functionally high-expressing mutant of rat neurotensin receptor 1”

Oct. 02 – Sept. 05 Bachelor Student at University of Zürich, Switzerland. Chemistry/Biophysics Track. Bachelor thesis conducted in the Department of Biochemistry under supervision of Prof. Dr. Amadeo Caflisch. Thesis title: “Fragment–based flexibel ligand docking”

Aug.95 – Jan.02 High School. Mathematisch-Naturwissenschaftliches-Gymnasium, Zürich, Switzerland

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