Regenerative therapies for intervertebral disc degeneration

*a translational approach to serve man and dog*

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Regenerative therapies for intervertebral disc regeneration
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Regenerative therapies for intervertebral disc degeneration

*a translational approach to serve man and dog*

Regeneratieve therapieën voor tussenwervelschijfdegeneratie

*Een translationele benadering om de mens en de hond te dienen*  
(met een samenvatting in het Nederlands)

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Chapter 1
General introduction, outline, and aims of this thesis

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Potential regenerative treatment strategies for intervertebral disc degeneration in dogs

Frances C. Bach, Nicole Willems, Louis C. Penning, Keita Ito, Björn P. Meij, Marianna A. Tryfonidou

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Low back pain and intervertebral disc (IVD) degeneration

Low back pain (LBP) is one of the leading causes of disability in the Western world, and is associated with high socio-economic costs due to increased morbidity of afflicted individuals, decreased productivity and increased health care costs. Approximately 84% of all people will experience an episode of LBP at some point in their lifetime; 50% of them at a young (18 – 44 years) or middle (45 – 64 years) age. In the Netherlands, the annual costs of LBP were estimated at € 3.5 billion in 2007, with 12% direct and 88% indirect costs respectively. After an initial period of LBP, in 44 – 78% of the patients a pain relapse has been described, and in 26 – 37% relapses of work absence. LBP can fluctuate over time with frequent recurrences and exacerbations, and 10% of the patients develop chronic persistent or recurrent pain, and generate approximately 80% of health care costs. Although the exact etiology of LBP remains still unclear, it has been described to be strongly linked to intervertebral disc (IVD) degeneration.

Current treatments

Conservative treatment

As the exact pathogenesis of IVD degeneration is still poorly understood, current treatments are mostly symptomatic, and have not been shown to allow for repair of the IVD thus far. Many clinical trials of variable methodologic quality have evaluated the therapy for nonspecific LBP. Cyclooxygenase-2 (COX-2) selective anti-inflammatory drugs (COX-2 inhibitors), muscle relaxants, physical therapy, spinal manipulation, and maintenance of ordinary activity have been shown to be effective for short-term pain relief in acute LBP and in patients with suspected disc herniation, without the cauda equina syndrome or progressive neurologic deficits. Some of the patients with disc herniation and radicular pain can benefit from epidural corticosteroid injections. In chronic LBP, various interventions, i.e. antidepressants, weak opioids, COX-2 inhibitors, cognitive behavioral therapy, supervised exercise therapy, brief educational interventions, back schools, and multidisciplinary (bio-psycho-social) treatment, have been shown to provide short-term effects on pain and function.

Surgical treatment

If pain persists despite conservative management, diagnostic imaging and subsequent surgical intervention can be considered. IVD degeneration can be assessed with several imaging modalities such as conventional radiography, computed tomography (CT), and discography. Thanks to the development of magnetic resonance imaging (MRI), this is nowadays the best diagnostic modality to detect changes of the IVD. In patients with lumbar disc herniation, the herniated disc fragment is removed by a minimally invasive procedure: a microdiscectomy. In patients with chronic symptomatic IVD degeneration, spinal fusion was the only surgical option for decades. To date, surgical fusion to treat
chronic LBP remains a controversial issue. Four randomized controlled trials have compared lumbar fusion to conservative treatment with conflicting results.\textsuperscript{23-26} Furthermore, fusion of a lower spinal segment alters the biomechanics of the rest of the spine, and predisposes patients to degenerative changes in the adjacent motion segments.\textsuperscript{27} In an effort to improve results of fusion and to reduce the occurrence of adjacent segment degeneration, total disc replacement techniques that preserve motion of the spine,\textsuperscript{28} have been developed and studied extensively. Nevertheless, clinical outcomes were equivalent to those with spinal fusion.\textsuperscript{21, 29-31} Unfortunately, in many patients, neither conservative nor surgical treatment results in a satisfying outcome.\textsuperscript{32} In order to design optimal strategies to biologically repair the IVD, it is important to know the healthy and diseased IVD.

**The healthy IVD**

IVDs are fibrocartilaginous structures embedded between the vertebral bodies, and provide stability and flexibility to the spinal column, by absorbing and transmitting mechanical loads.\textsuperscript{33} Each IVD is composed of a central well-hydrated proteoglycan-rich gel, the nucleus pulposus (NP), that is surrounded by concentric lamellae of alternating oblique collagen fibers, the annulus fibrosus (AF), and connected to the vertebral bodies by cartilaginous endplates (EP).\textsuperscript{33-35} The composition of the NP changes as the IVD matures: the number of large vacuolated cells of presumably notochordal origin decreases, whereas the number of smaller chondrocyte-like cells (CLCs) increases. As a result of the changes in cell types, the extracellular matrix (ECM) also changes.\textsuperscript{36} The major proteoglycan in the NP is aggrecan, which is embedded in a network of collagen type II and elastin fibers.\textsuperscript{34} Proteoglycans consist of a protein backbone with negatively charged glycosaminoglycan (GAG) side chains. The most common side chains are the anionic chondroitin sulfate and keratan sulfate, that are covalently bound to the core protein. Hyaluronic acid forms non-covalently linked complexes with proteoglycans, creating large negatively charged complexes that attract cations. This leads to water absorption and enables the NP to withstand large compressive forces.\textsuperscript{34, 35} The fibers in the outer part of the AF primarily consist of collagen type I, and are interconnected via elastic fibers, providing a firm network that is able to resist tensile forces and prevent separation of lamellae during compressive loading.\textsuperscript{33} The inner part of the AF is poorly organized and contains both collagen type I and II, and proteoglycans. Collagen fibers (Sharpey fibers) continue from the AF to the rims of the vertebral bodies, to the longitudinal ligaments anteriorly and posteriorly, and to the cartilaginous EP superiorly and inferiorly. The cartilage EPs lock into the osseous EPs via calcified cartilage.\textsuperscript{37} At birth the cartilaginous EPs and the peripheral AF are highly vascularized, while with aging a decrease in vascularization in both structures is described. The adult IVD becomes the largest avascular structure in the body, and relies on diffusion of nutrients from capillary blood
vessels in the subchondral bone through the cartilaginous EP, and to a lesser extent through vessels in the periphery of the AF. Innervation of the healthy adult IVD is provided by the sympathetic chain and the recurrent sinovertebral nerve, and is restricted to the outer layers of the AF.\textsuperscript{38}

**IVD degeneration**

During the process of IVD degeneration, the ECM in the IVD deteriorates as a result of mechanical trauma, injuries, smoking, obesity, and aging.\textsuperscript{34, 39-45} Mechanisms that may contribute to this deterioration include inadequate nutrient supply, reduced cell viability, cell senescence, and programmed cell death.\textsuperscript{46-49} IVD degeneration is characterized by elevated levels of inflammatory cytokines, increased proteoglycan (aggrecan) and collagen type II degradation in the NP, and alterations in IVD cell phenotypes.\textsuperscript{35, 37, 50} Matrix metalloproteinases (MMP1, MMP-2, MMP-3, MMP-8, MMP-9), and aggrecenases (a disintegrin and a metalloprotease with thrombospondin motifs (ADAMTS)-1, ADAMTS-4, ADAMTS-5, ADAMTS-9, and ADAMTS-15) are thought to play a fundamental role in the degradation of collagens and proteoglycans within the ECM of the IVD.\textsuperscript{51-54} Due to the loss in proteoglycans, the IVD loses its hydrostatic properties, promoting structural wear of the IVD. Consequently, the normal function and stability of the motion segment, comprising the IVD, facet joints, and the adjacent vertebral bodies, changes, resulting in decreased disc height, osteophyte formation, facet joint arthritis, and deformation of vertebral bodies.\textsuperscript{34, 55} These structural changes and instability are strongly associated with painful pathologies, i.e. sciatica, disc herniation, and spinal stenosis. However, a majority of individuals over 30 years of age have some structural degenerative changes of one or more IVDs on MRI, but do not experience pain. Most probably pain is evoked when a structural deficit is accompanied by a secondary event, such as leakage of NP material through AF fissures, that results in attraction of immune cells and triggers a nociceptive response in the AF and/or the dorsal longitudinal ligament.\textsuperscript{50, 56, 57}

**Role of inflammatory molecules in IVD degeneration**

Thus far it is unclear if IVD degeneration starts with the aforementioned molecular changes in the ECM of the NP and AF that trigger the inflammatory response,\textsuperscript{58, 59} and/or if structural deficits in the NP and the AF, i.e. clefts, tears, herniation, enable recruitment of immune cells to the IVD (Figure 1).\textsuperscript{60, 61} Chemotactic mechanisms may play a crucial role in IVD degeneration and repair, as AF cells can be recruited by chemokines, and chemokine receptors have been identified on both NP and AF cells.\textsuperscript{62, 63} Although a physiological inflammatory response to sterile tissue injury primarily serves to promote tissue repair, an excessive inflammatory response with detrimental effects on tissue integrity, might contribute to the pathogenesis of IVD degeneration.\textsuperscript{50}
Figure 1. Schematic representation of the inflammatory response within the intervertebral disc (IVD). Causes of IVD degeneration are likely to be both genetic and environmental (initiating factors). IVD degeneration is mediated by increased levels of pro-inflammatory molecules secreted by nucleus pulposus (NP) and annulus fibrosus (AF) cells. Several inflammatory mediators (including TNF-α, IL-1β, PGE₂) can trigger a range of pathogenic responses in the NP and AF resulting in a catabolic environment. Molecular and biochemical changes in the ECM of the NP and AF further enhance inflammation, which in combination with structural deficits in the NP and AF enable recruitment and infiltration of the IVD by macrophages, neutrophils, and T cells.
Regardless of predisposing factor(s) or age, IVD degeneration is thought to be influenced by increased levels of pro-inflammatory molecules secreted by NP and AF cells, as well as macrophages, T cells, and neutrophils. Several of these molecules have the capability to trigger a range of pathogenic responses in NP and AF cells, and have been suggested to play a role in the catabolic processes in degenerated IVDs. Increased expression of IL-1β and a disturbance of the balance between the activating receptor of interleukin 1 (IL-1R), and the IL-1 receptor antagonist (IL-1Ra), has been demonstrated in degenerated IVD tissue. Tumor necrosis factor alpha (TNF-α) and IL-1 induced upregulation of matrix degrading enzymes by NP cells. Furthermore, elevated levels of IL-1 and prostaglandin E2 (PGE2) have been associated with aging and degeneration of the IVD. In NP cells, PGE2 negatively affected matrix integrity by inhibiting proteoglycan synthesis, possibly mediated by a decrease in insulin growth factor 1 and an increase in matrix degrading enzymes. Higher expression levels of interferon gamma (IFN-γ), IL-6, and IL-17 have been described in degenerated human IVDs compared with non-degenerated control IVDs. In response to elevated TNF-α and IL-1β levels, the expression of the chemoattractive protein chemokine (C-C motif) 3 (CCL3) in NP cells correlated positively with the grade of IVD degeneration. Furthermore, expression of CCL2, CCL7 and IL-8 increased concordant with histological degenerative changes in the NPs of degenerated IVDs.

Herniated degenerative IVD tissue has been demonstrated to spontaneously produce increased amounts of MMP-2, MMP-3, and MMP9, nitric oxide, PGE2 and IL-6, and increased gene expression levels of IL-1, IL-8, and TNF-α in vitro. TNF-α, and IL-8 levels were significantly higher in herniated IVDs compared with those in degenerated IVDs, while no significant differences were observed in the expression of IL-1β and IL-6 levels. In another study, IFN-γ, IL-4, IL-6, IL-12, and IL-17 levels were significantly higher in herniated IVDs compared with those in non-degenerated IVDs, whereas IFN-γ and IL-6 levels were higher in herniated IVDs compared with those in degenerated IVDs. These data suggest that the inflammatory reaction in herniated IVDs is distinct from that observed in degenerated IVDs. In addition, concentration of cytokines and cytokine profiles might also differ for different types of disc herniation.

Pain associated with IVD degeneration is believed to be related to ingrowth of small non-myelinated nerve fibers into the usually aneural areas of the IVD, and nociceptive stimulation of the surrounding neural elements due to IVD dysfunction. The release of cytokines, particularly IL-1β, has been shown to induce significant increases in nerve growth factor (NGF) and vascular endothelial growth factor, which could stimulate innervation and vascularization in the degenerated IVD. Furthermore, NGF is produced by newly formed microvessels, deriving from adjacent vertebral bodies.
General introduction, outline, and aims of this thesis

Ex vivo and in vivo animal models to study human IVD degeneration
Commonly used in vitro cell culture models in IVD research include 2D monolayers, 3D high density culture and alginate beads. A major disadvantage of these systems is that cells are removed from their native tissue environment during cell isolation, which might affect cell behavior. Hence, organ culture bioreactor systems have been developed to mimic the in vivo situation. IVD explants, including NP, AF and EPs can be kept alive under loading conditions for several weeks with preservation of tissue integrity. Despite these valuable alternatives and their contribution to a reduction in animal experiments, these models have clear limitations regarding the complex biology underlying IVD degeneration. Several animal models in IVD degeneration research have been developed to investigate the etiopathogenesis and treatment of the degenerative process, and to perform safety studies, prescribed and dictated by regulatory bodies.

Frequently used small animal models include rodent and rabbit models, and large animal models are dog, sheep, and goat models. In most animal models IVD degeneration is induced surgically or chemically. Only few animals develop spontaneous IVD degeneration, i.e. sand rats, pintail mice, baboons, and dogs. There are many differences in size, cell type, biochemical and biomechanical properties between human and animal IVDs, and the ideal translational model remains undetermined. Although induced IVD degeneration can result in a useful acute model of disc degeneration, it is likely that pathological pathways differ from the chronic condition of IVD degeneration in humans. Variations in anatomical dimensions between species, (e.g. size, shape, adjacent spinal tissues) obviously affect the biomechanical behavior of the spinal segments and make a direct comparison problematic. The presence of notochordal cells (NCs) differs between various species and has been suggested to initiate results that are more favorable in promoting regeneration and repair. In humans, cows, sheep, and horses these cells disappear with aging, while in pigs, rabbits, mice, and rats, they persist up to adulthood. Interestingly, in humans and chondrodystrophic dogs, the loss of NCs from the NP occurs before early signs of degeneration, and it remains unclear if their disappearance might play a role in initiating IVD degeneration.

The dog as a patient
The dog is the only large animal model with naturally occurring IVD degeneration, which is also clinically treated for IVD disease in its role as a companion animal. In chondrodystrophic (CD) dog breeds (e.g. dachshund), endochondral ossification of the long bones is disrupted, resulting in disproportional short limbs. Chondrodystrophy is
believed to be closely linked to the occurrence of IVD degeneration with concurrent loss of NCs at all IVD levels. By 1 year of age, this transformation is complete in 75% of the cervical, 100% of the thoracic, and 93.8% of the lumbar IVDs, and ultimately affects all IVDs. In contrast to CD dogs, in non-chondrodystrophic (NCD) dogs IVDs remain healthy with high numbers of NCs until late in life. Degeneration of IVDs associated with loss of NCs in NCD dogs occurs at predisposed sites, i.e. the caudal cervical and lumbosacral (LS) spine, at 6 to 8 years of age. Lifetime prevalence of IVD degenerative diseases in CD and NCD dog breeds are calculated at 20% and 7%, respectively. CD dogs are predisposed to explosive extrusion of the NP (Hansen type I) of degenerated lumbosacral and caudal cervical IVDs, and NP extrusion of degenerated thoracolumbar IVDs. Hansen type II annular protrusion does occur in CD dogs, but less commonly. The overall case fatality rate (rate of mortality to incidence rate of IVD-related diseases) in CD and NCD dogs is 25% and 65%, respectively. As in humans, canine IVD degeneration is associated with cervical and (low) back pain and neurologic deficits.

Current treatments for IVD disease in painful dogs focus on alleviating pain and include analgesics (e.g. anti-inflammatory drugs, opioids, gabapentin), muscle relaxants, and physical therapy. In dogs with neurological deficits, or in dogs that do not respond to conservative treatment, diagnostic imaging is indicated, e.g. CT or MRI, and surgical intervention is recommended. Aim of the surgery is to decompress affected neural thoracolumbar and cervical IVDs, mainly between 3 and 7 years of age. NCD dogs are predisposed to protrusion of the AF (Hansen type II) of degenerated structures. Several direct decompression procedures are routinely performed in veterinary practice, such as dorsal laminectomy in dogs with degenerative lumbosacral stenosis (DLSS), hemilaminectomy in dogs with thoracolumbar IVD disease, or a ventral slot-procedure in dogs with cervical IVD disease. Indirect decompression techniques have also been described, e.g. distraction-stabilization-fusion in dogs with DLSS and caudal cervical spondylomyelopathy. In the last few decades pedicle screw-rod fixation (PSRF) in canine patients have gained interest to treat lumbosacral spinal stenosis, instability and degenerative disc disease. Nevertheless, long-term side-effects of medication, spinal instability, and recurrence of IVD disease are commonly described in dogs. Similar to the situation in human medicine, current therapies in dogs do not restore functionality of the degenerative IVD, and a clear need exists for development of regenerative therapies.

The dog as a translational animal model
A collaboration between biomedical researchers and veterinary surgeons may be beneficial to both humans and dogs. Dogs are an interesting species in IVD research, as they can be divided into CD and NCD breeds, based on their physical appearance. Similar to IVD degeneration in humans, NCs in the NP of CD dogs are gradually replaced by CLCs
early in life. As a result, already by one year of age, the NP of CD dogs contains primarily CLCs, while NCs remain the predominant cell type in the NP of NCD dogs during their lifetime.\textsuperscript{87, 103, 104} A few \textit{in vivo} studies investigating regenerative therapies for the IVD have been performed in laboratory dogs with experimentally induced IVD degeneration.\textsuperscript{105-108} Interestingly, dogs with spontaneously occurring IVD degeneration have not been used in such \textit{in vivo} studies thus far. Beagle dogs (CD breed), represent a uniform population with naturally occurring IVD degeneration, and can be useful in testing regenerative strategies for early and intermediate stage IVD degeneration.

Companion dogs, that naturally develop IVD degeneration and disease, may serve as appropriate candidates for preclinical trials of innovative treatments for intermediate or late stage IVD degeneration. Clinical outcomes can be evaluated by physical examination, diagnostic and quantitative imaging, completed questionnaires by owners, and gait analysis. Canine patients will probably benefit from such trials in future, as these innovative therapies would otherwise not be available in veterinary medicine, while biomedical researchers can profit from the outcome of a representative animal model, testing the feasibility of regenerative treatments, prior to conducting clinical trials in human patients.

**Intrinsic IVD regeneration**

Evidence is increasing that the IVD itself is populated with resident progenitor cells in the NP, AF, and endplate in young and aged IVDs of different mammals (human, dog, macaque, minipig, rat, mouse).\textsuperscript{109-116} It is suggested that the resident stem cells within the IVD are remnants of the multipotent mesoderm cells (notochord) during embryogenesis.\textsuperscript{117, 118} However, it has recently been shown that other stem cells migrate from specific niches localized in the outer AF borders with the ligament zone and the perichondrium, towards the inner parts of the IVD.\textsuperscript{110} Limited numbers of intravenously delivered bone marrow derived mesenchymal stem cells (MSCs) were also shown to be able to migrate to the IVD,\textsuperscript{119} suggesting MSCs originating from the vertebral bone marrow might also serve as a resource of IVD stem cells. During IVD degeneration, the intrinsic repair capacity might be inadequate, or might be disturbed by inflammatory and/or catabolic processes that accelerate ECM degradation.\textsuperscript{60, 64-67} Even more so, \textit{in vitro} differentiation of IVD derived stem cells was shown to induce to expression of neural or endothelial markers, facilitating vascularization and innervation of the IVD.\textsuperscript{112, 120}
Current regenerative approaches

The current clinical therapeutic limitations, in combination with a rapid increase in understanding of the etiopathological background of IVD degeneration, stimulated many researchers to investigate novel regenerative therapies (Figure 2). Regenerative treatments aim at intervention at an early stage of IVD degeneration, and comprise restoration of the homeostasis of the ECM, control of inflammation, and prevention of angio- and neurogenesis. Intradiscal delivery of exogenous growth factors, genes, and cells, either alone or in combination, are promising regenerative approaches. To optimize cell activity, viability, preservation, and differentiation down the desired mesenchymal lineages, regenerative strategies also focus on optimal cell carriers and delivery systems, such as biomaterials. Thus far, novel regenerative therapies have been tested in animal models with induced IVD degeneration, but not in animal models with spontaneous IVD degeneration that more closely resembles the biological condition in humans.

Figure 2. A midsagittal histologic section (picrosirius red/alcian blue) of a healthy (upper left), and a degenerated human IVD (upper right). The healthy IVD consists of a nucleus pulposus (NP) rich in collagen type II and proteoglycans (blue stain), and a well-organized fibrocartilaginous annulus fibrosus. The degenerated IVD consists of a NP rich in collagen type I (red stain), a highly disorganized AF, and is reduced in height. Intradiscal delivery of growth factors, anti-inflammatory factors, biomaterials, and cells, and (temporary) biomechanical adjustment of a spinal segment, either alone or in combination, seem promising regenerative strategies for IVD repair.
A relatively new approach to cartilage regeneration, that originates from the field of osteoarthritis, is joint distraction.\textsuperscript{122, 123} This surgical technique is based on the hypothesis that osteoarthritic cartilage has some regenerative capacity when the damaged cartilage is mechanically unloaded by means of an external fixation frame. In this way further wear and tear is prevented, while the intermittent synovial fluid pressure, essential for the nutrition of the cartilage, is preserved.\textsuperscript{124} The exact mechanisms that lead to cartilage regeneration in the distracted joint space are not known, but are currently being investigated. Because of the large similarities between articular cartilage and the IVD, several studies have focussed on the effects of segmental distraction in IVD disease.\textsuperscript{125, 126}

**Growth factors**

Several growth factors have been shown to effectively promote cell production and cell proliferation of IVD cells \textit{in vitro} as well as \textit{in vivo}. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta superfamily. Transforming growth factor beta (TGF-\(\beta\)) is one of the first applied growth factors that was shown to stimulate IVD cell proliferation.\textsuperscript{65, 127, 128} In a mouse IVD compression model TGF-\(\beta\) stimulated proliferation of AF cells, which also showed an increased gene expression of aggrecan and collagen II.\textsuperscript{129} Importantly, response to TGF-\(\beta\) was observed only after administration of multiple injections, indicating a need for sustained delivery.

\textit{In vitro} and \textit{in vivo} studies have indicated that BMP-2 and BMP-7, also members of the TGF-\(\beta\) superfamily, are upregulated with aging and with induced IVD injury.\textsuperscript{130-135} BMP-2 was shown to enhance matrix production and increase gene expression of collagen type II and aggrecan in rat, rabbit and human IVD cells.\textsuperscript{136-140} IVD cells from 6-month-old and 3-year-old rabbits that were treated with BMP-2 showed increased synthesis of GAGs and increased gene expression of aggrecan, collagen type I and II. Interestingly, this effect was more pronounced in adult rabbit cells compared with the adolescent IVD cells.\textsuperscript{141} Similar results were demonstrated in alginate-encapsulated bovine NP cells that were co-cultured with transduced bovine cartilage cells expressing BMP-2.\textsuperscript{142} Results on the regenerative effects of BMP-2 on degenerated IVDs \textit{in vivo} are conflicting. Intradiscal injection of adeno-associated virus serotype 2-BMP in a rabbit annular puncture model resulted in slowing the course of injury-induced degeneration. However, in another rabbit annular puncture model no regenerative effects were reported after injection of transfected IVDs cells expressing BMP-2.\textsuperscript{143} In a rabbit annular tear model, BMP-2 protein provoked acceleration of IVD degeneration, and osteogenic responses were observed near the vertebral endplates.\textsuperscript{144}

BMP-7 (also known as osteogeneic protein-1) has been widely studied for its regenerative effects on degenerated IVDs. \textit{In vitro}, BMP-7 has been shown to enhance ECM production
of rabbit, bovine, and human IVD cells. Recombinant human (rh)BMP-7 was demonstrated to restore disc height and increase the proteoglycan content in a rabbit annular puncture model, and to have anti-catabolic effects in a rat IVD compression model. In a canine model of allogenic IVD transplantation, NP cells expressing hBMP-7 prevented degeneration of the transplanted IVD at 6 months follow-up compared with non-transfected NP cells.

In growth and differentiation factor 5 (GDF-5; also known as BMP-14) deficient (GDF -/-) mice, reduced levels of GAGs, and downregulation of type II collagen and aggrecan genes in the IVDs were reported. Stimulation of bovine NP and AF cells with rhGDF-5, enhanced cell proliferation, and proteoglycan and collagen synthesis and accumulation. In vivo, a single injection with rhGDF-5 into the NP was shown to improve disc height, MRI and histological grading scores in a rabbit annular puncture model. Sustained release of rhGDF-5 from PLGA microspheres also resulted in restoration of disc height, increased GAG and DNA content, and significantly increased mRNA levels of collagen type II in a rat annular puncture model. Several clinical trials in which a single dose of rhGDF is intradiscally injected are ongoing (NCT01158924, NCT01182337, NCT01124006, NCT00813813), however, outcome data have not been published yet.

Various in vitro studies have proven that insulin-like growth factor-1 (IGF-1) promotes cell proliferation and matrix synthesis. Platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and epidermal growth factor enhanced IVD cell proliferation. IGF-1 and PDGF were also shown to reduce the percentage of apoptotic human AF and in rat NP cells. Only minor effects on cell proliferation of IGF-1 and basic fibroblast growth factor in a mouse IVD compression model were reported.

Platelet-rich plasma (PRP), a natural carrier of multiple growth factors has also been introduced in the field of IVD regeneration. Multiple growth factors and other proteins in this therapeutic cocktail are thought to be synergistic. In several in vitro studies PRP was shown to induce proliferation of NP and AF cells, and differentiation of NP cells. A few preclinical studies in rat and rabbit annular puncture models show promising data on regeneration of the IVD by a single intradiscal injection of PRP. Nevertheless, further studies are required to confirm the efficacy and safety of intradiscal application of PRP, considering the potential undesired effects of vascularization, ossification and inflammation.
Cell-based therapies
Since there are relatively few cells in the degenerated IVD, i.e. less than 1% of the total disc volume, and cell viability is impaired, stimulation of the residing cells may be insufficient to achieve repair. Supplementation of functional cell populations may overcome this problem. Cells used in IVD tissue engineering should be able to survive within the local environment, and produce a functional matrix, which is similar to the original tissue. Thus far, cell-based treatment strategies have mainly focussed on NP cells, articular chondrocytes, and MSCs have found their way to the clinic.

Disc derived chondrocytes and articular chondrocytes
Transplantation of autologous and allogeneic NP and/or AF cells into the IVD has been shown to retard IVD degeneration in various species. A few clinical trials have been performed using cell-based therapies for IVD repair. In the Euro DISC study, culture-expanded autologous IVD cells harvested during discectomy in humans, were transplanted via a minimal invasive percutaneous injection. Two-year follow-up data demonstrated a significant pain reduction, preservation of disc height, and maintained hydration of adjacent segments in the cell treated group compared with the discectomy only group. However, this study was not placebo-controlled, therefore results should be interpreted with care. In the NuQu phase I safety study, allogeneic juvenile knee chondrocytes were percutaneously injected into degenerated lumbar IVDs of 15 human patients and evaluated after 12 months. Pain scores significantly improved, and the majority of radiological parameters improved or remained unchanged. An ongoing phase II clinical trial will assess the safety and efficacy of this approach.

Mesenchymal stem cells (MSCs)
MSCs are emerging as leading cellular therapy for several diseases, since they can easily be isolated from a variety of tissues, e.g. bone marrow, adipose and synovial tissue, and can differentiate into different cell types, including chondrocytes. Several in vivo studies in rabbit, rat, and goat models have demonstrated that bone marrow derived MSCs (BMSCs) are able to maintain their viability, proliferate, and obtain an IVD-like phenotype after implantation into the IVD. Intradiscal delivery of BMSCs and adipose derived stem cells (ASCs) has been shown to promote regeneration in an experimentally induced IVD degeneration models in dogs. Furthermore, human MSCs survived after implantation into porcine models, and expressed typical chondrocyte markers, suggestive of differentiation toward IVD-like cells. Several clinical trials (phase I and II studies) with autologous and allogeneic MSCs are currently being performed (NCT01860417; NCT02338271; NCT01290367).
**Biomaterials**

Biomaterials are commonly used as cell carriers, however, they can also improve the effectiveness of bioactive agents, e.g. growth factors, and pharmaceutical agents, by sustained release. Biomaterials are recognized for their biocompatibility, biofunctionality, and bioresorbability, and they can be delivered into the IVD via minimally invasive procedures (Figure 3). By encapsulating bioactive substances in a biomaterial, the loaded compound can be protected, and bioavailability and stability can be increased. Furthermore, higher loading doses of pharmaceutical agents can be achieved, without causing local and systemic side effects, as they are released locally, and over a longer period of time.

**Hydrogels as cell carriers**

Hydrogels represent an important class of biomaterials, and have been employed for a range of biomedical applications. Hydrogels are networks of hydrophilic polymers, and have the capacity to absorb large amounts of water or biological fluids. Crosslinkages in...
these networks can be based on covalent (e.g. photo-crosslinked), or on physical (e.g. hydrophobic) interactions. Formation of a hydrogel delivery system upon injection of combinations of reactive compounds, can avoid procedures that may harm the cells and bioactive substances (e.g. photo crosslinking). Different mild crosslinking chemistries, e.g. Schiff’s base chemistry, and Michael-type reactions, can be used under physiological conditions to form networks and encapsulate cells within the gel. In studies concerning these type of hydrogels, cell viability of mesenchymal progenitor (MPCs) and stem cells, NP, and AF cells and proteoglycan production were retained in vitro.187-191

**Natural hydrogels**

Hydrogels consisting of natural polymers, e.g. collagen and hyaluronic acid (HA), are commonly used as cell carriers to retain transplanted cells at the site of injection. They show analogy with the disc microenvironment, which facilitates survival of the transplanted cells, or even promotes matrix production. Protease solubilized collagen (Atelocollagen®) has been shown to be less immunogenic compared with other carriers, and to enable cell proliferation, matrix synthesis and differentiation of MSCs in a rabbit induced degeneration model.180 HA in combination with ASCs has been shown to maintain IVD morphology and disc height on MRI, in a canine induced degeneration model, suggestive of preservation of cell viability and production of ECM matrix.107 An HA-based hydrogel (Durolane®) induced the highest cell proliferation of human MSCs, chondrocytes, and IVDs in vitro. Interestingly, intradiscal injection of this HA-based hydrogel in a porcine degeneration model, either with or without human MSCs or chondrocytes, resulted in bone formation in the IVD at 6 months follow-up, indicating its unsuitability for use in vivo.192 In an ovine chemonucleolysis model, IVD degeneration scores and disc height index (DHI) significantly improved, when injected with an HA carrier containing MPCs, compared with non-injected and HA-injected IVDs.193 Currently, two clinical trials are being performed, in which HA is used as a carrier for autologous MPCs (NCT02338271; NCT01290367).

**Synthetic hydrogels**

Polyethylene glycol (PEG) is a synthetic polyether which is rarely used as a stand-alone hydrogel in regenerative strategies because of their bio-inert nature. However, in combination with adhesive peptides or other hydrogels they do support cell adhesion and tissue formation.194 PEG hydrogels are not naturally degradable, unless combined with synthetic or natural components such as poly lactic acid (PLA) or enzyme-sensitive peptides.194, 195 Recently, a PEG-crosslinked serum albumin/HA hydrogel was shown to support cell viability and chondrogenic differentiation of articular chondrocytes, IVD cells and MSCs, and to have anti-angiogenic properties in vitro and in vivo.196, 197 In a nucleotomy-induced IVD degeneration model in sheep, this hydrogel was combined with...
IVD cells, and was shown to enhance the process of endogenous repair. A prospective randomized clinical trial was started in humans to evaluate the clinical applicability, safety and efficacy of this hydrogel combined with autologous IVD chondrocytes in the repair of a herniated disc with an indication for an elective sequestrectomy (NCT01640457).

**In situ forming hydrogels using thermoresponsive systems**

Physically crosslinked hydrogels can be formed via non-covalent interactions in response to a change in temperature. Injectable thermoreversible hydrogels are of great interest in the field of regenerative repair of the IVD, as formation in situ occurs in a mild way, without the need of chemical reactions, and moulding of the hydrogel into the exact shape of the defect is easy. Natural (e.g. chitosan, alginate) as well as synthetic polymers, (e.g. poly-N-isopropylacrylamide (pNIPAAM)) gels are frequently used. These gels can serve as delivery systems because of their ease of control and preparation, and practical application; they can easily be injected into the IVD as a fluid, and undergo a phase transition to an insoluble state with increasing temperature, diminishing the risk of extrusion or leakage from the IVD. Several thermoreversible hydrogels, e.g. HA-pNIPAAM, and chitosan-glycerophosphate (C/GP), supported differentiation of human MSCs toward the IVD phenotype without the need for growth factor supplementation *in vitro* and *ex vivo*. NP cells embedded in a C/GP thermosensitive hydrogel produced proteoglycans that were retained in the chitosan-matrix, whereas AF cells did not survive the hydrogel formation process.

**Biomaterials as sustained delivery agents**

Bioactive substances can be incorporated into hydrogels by simple mixing, or encapsulated in microspheres, allowing accurate and sustained delivery of the active compounds via intradiscal injection. To avoid problems with variability of production, mechanical properties and degradation rates of natural polymers, networks can be completed or created with synthetic polymers, e.g. PLA, PEG, or polyglycolic acid, resulting in formation of hydrogels or microspheres that can easily be tuned and reproduced, and degrade in a controlled fashion. Thus far only limited studies *in vitro* and *in vivo* are available on sustained release in the IVD, targeting different aspects (e.g. inflammation, imbalance anabolism/catabolism) of IVD degeneration.

**Hydrogels**

A ferulic acid (FA) loaded chitosan-gelatin-glycerophosphate (C/G/GP) hydrogel has been shown to provide sustained release of FA, resulting in a reduction in cell apoptosis, and anabolic effects on gene expression and protein levels *in vitro*. Biodegradable gelatin hydrogel microparticles loaded with platelet rich plasma were shown to suppress
progression of IVD degeneration, maintain disc height, and increase expression of anabolic genes in an induced IVD degeneration model in rabbits.205, 206

**Microspheres**

The release medium of IL-1Ra encapsulated in poly lactic-co-glycolic acid (PLGA) microspheres attenuated degradative effects of IL-1β in bovine NP cell constructs. However, decreased efficacy of the encapsulated protein was demonstrated at later time intervals, most likely due to a diminished concentration of released IL-1Ra with time and/or potential reduced bioactivity.207

Sustained release rhGDF-5 from PLGA microspheres was associated with restoration of disc height, increased GAG and DNA content, and increased mRNA levels of collagen type II, in punctured IVDs in a rat tail degeneration model. As a single injection with rhGDF-5 was not included in this study, the added value of sustained delivery could not be demonstrated.158

Positively charged PLGA microspheres loaded with dexamethasone combined with negatively charged nanoparticles loaded with bFGF-embedded heparin/poly(l-lysine) showed in vitro growth of MSCs and chondrogenic differentiation.208 Injection of these microspheres combined with TGF-β3-embedded heparin/poly(l-lysine) nanoparticles, and seeded with ASCs in a rat IVD degeneration model, resulted in a significant increase in proteoglycan production compared with controls, and a restored disc height of 70% of the healthy controls.209 No additive effects of the seeded ASCs were shown, indicating results were mediated by stimulation of cells in situ. Ceramic capsules loaded with corticosteroids significantly reduced cellular degeneration in a rat model, when placed adjacent to the injured IVD, and the authors suggested a beneficial effect of corticosteroids on remodelling of the IVD.210

**Joint distraction**

As stated before, in many patients neither conservative nor surgical treatment results in a satisfying outcome.211 A promising technique in peripheral osteoarthritic (OA) joints that preserves function without major surgery is distraction.212 Distraction of a joint by using an external fixator has been shown to decrease mechanical stresses on cartilage, and to initiate repair by chondrocytes. Although the IVD and articular cartilage morphologically seem very different, they are remarkably similar at a biochemical level.213 Furthermore, in parallel to OA joint cartilage, the IVD is also subjected to mechanical stress. Distraction of both the IVD and the facet joints can be established by using a PSRF construct that can be placed with a minimally invasive surgical technique. In human cadavers lumbar distraction appeared to predictably reduce NP pressure.214 This type of construct has been used
extensively in spinal fusion to provide biomechanical stability to obtain fusion, to decompress neural structures and to correct deformities. However, spinal fusion alters the biomechanics of the spinal column, and it is thought that the loss of motion at the fused level leads to increased motion and load at the unfused segments, resulting in adjacent segment disease (ASD). Placing a fixation device only temporarily to provide distraction, might avoid development of ASD, but allow biological repair of the IVD. Several studies focused on the dynamic and biochemical effect of temporary joint distraction in the treatment for IVD degeneration by using a rabbit dynamic distraction model. In this model, signs of tissue repair were demonstrated at a biological, cellular and biomechanical level after 28 and 56 days of external disc distraction. Also, regeneration of the ECM in the NP and EPs was described, as well as regeneration of vascular channels in the EPs. Furthermore, rehydration of the IVD, stimulation of ECM gene expression, and increased numbers of BMP-2 and collagen type II positive cells were shown. Although, these results demonstrate the potential benefit of temporary IVD distraction on the regenerative capacities of the IVD, further studies are warranted before this technique can be applied in clinical practice.

**Clinical imaging of IVD regeneration**

Regenerative therapies are shifting towards prevention and intervention at early stages, and accurate, objective, and non-invasive assessment of the condition of the matrix, and the effectiveness of treatment in *in vivo* studies is required. The most frequently used MRI techniques for imaging spinal structures are T1-weighted (T1W) and T2W images. High intensity areas in T1W images indicate a high fat concentration. This fat acts as a natural contrast medium, and structures bordered by fat are clearly outlined. The bright signal intensity of water predominates in T2W images and is highly significantly correlated with the water and proteoglycan content, and hence negatively correlated with the extent of IVD degeneration. The Pfirrmann classification system is the most widely used system to grade human IVD degeneration on T2W images in research and clinical applications. The system evaluates T2W signal intensity, IVD structure, distinction between NP and AF, and disc height. Conventional imaging techniques have limited capabilities in revealing subtle changes at the ECM level and therefore novel techniques have been developed. T2W images provide only qualitative data, whereas with quantitative techniques, e.g. T2 mapping, relaxation times are calculated, that provide information about tissue structure. The T2 relaxation time is characterized by a time constant (T2) representing the decay of the transverse magnetization towards zero after a radiofrequency pulse. T2 relaxation time correlates positively with hydration and negatively with the composition of the collagen network structure. Several studies have demonstrated decreased T2 relaxation times in the NP from sagittal maps, when Pfirrmann scores increased.
A technique that has gained interest because of its supposed capacity to identify early degeneration by the loss of proteoglycans is $T1p$. $T1p$, the time constant that is obtained by spin-lock MRI, is similar to $T2$ in that it is sensitive to interactions of water with macromolecules.\textsuperscript{224, 236, 237} However, the spin locking makes $T1p$ more sensitive to different relaxation mechanisms, such as low frequency proton exchange. This leads to an enhanced dynamic range of $T1p$ compared with $T2$, and enables the detection of changes in proteoglycans.\textsuperscript{238, 239} $T1p$ relaxation time has been shown to be directly correlated to proteoglycan content and (inversely) to degenerative grades in the IVD.\textsuperscript{240-243} Mean $T1p$ values in the NP have been demonstrated to be significantly higher compared with $T1p$ values in the AF.\textsuperscript{244} Interestingly, a significant correlation between $T1p$ values and clinical symptoms has been reported. Nevertheless, further studies are warranted, as this study was performed in only 16 patients.\textsuperscript{243}

In nuclear magnetic resonance spectroscopy (MRS), molecular groups that carry hydrogen nuclei can be visualized, as the exact frequency at which a proton resonates depends on the chemical bonds the atoms form.\textsuperscript{224} MRS studies in IVDs \textit{ex vivo} have shown to be able to detect a decrease in proteoglycan content in the NP and an increase in collagen degradation levels with increased degeneration.\textsuperscript{245, 246} Feasibility of the quantification of water and proteoglycan content \textit{in vivo} by MRS was already demonstrated,\textsuperscript{247} and future developments may allow \textit{in vivo} evaluation of other metabolite resonances, increasing the possibility of exploring the chemical condition of the IVD.
Outline and aims of this thesis

The main aim of this thesis is to develop innovative treatments to regenerate early degenerated IVDs in order to regain function and thereby prevent or delay further medical intervention.

Therefore, bioactive substances are delivered intradiscally, either alone, or in combination with injectable sustained release systems, and are evaluated in a canine model predisposed to spontaneous IVD degeneration. Furthermore, distraction of the lumbosacral junction is investigated in an in vivo pilot study.

Aim 1: determine the safety of intradiscal injection and biocompatibility of PEAMs in a canine IVD degeneration model.

Rationale: Within the IDiDAS project (New Early Therapies for Intervertebral Disc Diseases. Drug Delivery and Augmentation through Smart Polymeric Biomaterials), which forms part of the research program of the Biomedical Materials institute (BMM), two injectable sustained release systems, i.e. polyester amide microspheres (PEAMs) and pNIPAAM MgFe-LDH hydrogel have been developed for intradiscal application. Extensive studies demonstrated sustained release and bioactivity of small molecules of these sustained release systems in vitro.

Approach: The safety of intradiscal injection and biocompatibility of PEAMs is investigated in a canine early IVD degeneration model in chapter 2. Safety of this treatment is assessed by magnetic resonance imaging in vivo and at a biochemical, biomolecular, and histopathological level post mortem. Safe intradiscal application will support further in vitro and in vivo studies on degradation and release profiles of PEAMs loaded with bioactive substances, including growth factors.

Aim 2: Evaluate the efficacy and safety of intradiscal application of rhBMP-7 in a canine IVD degeneration model.

Rationale: Studies in rabbit models with induced IVD degeneration demonstrated a regenerative effect of only one single injection of rhBMP-7 on the IVD.

Approach: As a first step to translate a treatment strategy based on growth factors towards a clinical application, in chapter 3 we investigate whether a single injection of rhBMP-7 will be safe and have similar effects in an animal model with spontaneous IVD degeneration. Based on the most effective and safe dose, follow-up studies can concentrated on developing controlled release systems for the sustained delivery of rh-BMP-7.
Aim 3: Evaluate the efficacy and safety of sustained delivery of an anti-inflammatory drug by an intradiscally injected hydrogel in a canine IVD degeneration model.

**Rationale:** As inflammation is thought to play an important role in the process of IVD degeneration and disease, sustained release of an anti-inflammatory drug is investigated to suppress inflammation and to restore IVD homeostasis. *In vitro* studies showed prolonged inhibition of inflammation when applying a pNIPAAM MgFe-LDH hydrogel loaded with a specific COX-2 inhibitor, celecoxib (CXB), compared with a CXB bolus formulation.

**Approach:** First, safety and efficacy of sustained release of a low dose of CXB from this hydrogel is evaluated after intradiscal injection in the canine spontaneous IVD degeneration model. After confirming safety, a dose-response study is performed to determine the optimal loading dose of CXB (*chapter 4*).

Aim 4: Investigate inflammatory profiles in canine intervertebral disc degeneration.

**Rationale:** The clinical representation of IVD disease in veterinary medicine is diverse. Chondrodystrophic and non-chondrodystrophic dogs present with clinical IVD disease at different spinal locations and at different ages.

**Approach:** In order to select canine patients that will benefit most of sustained release of CXB, we determine inflammatory mediators and matrix components in IVD samples that were collected during surgical treatment from CD and NCD dogs with and without clinical signs in *chapter 5*.

Aim 5: Assess long-term outcome of treatment of dogs with degenerative lumbosacral stenosis (DLSS) treated with pedicle screw-rod fixation (PSRF)

**Rationale:** Surgical management is the treatment of choice for dogs and humans with refractory symptoms of degenerative lumbar stenosis. Although the safety and efficacy of PSRF for the treatment of DLSS in large breed dogs has been established, long-term results and evaluation of spinal fusion were not reported thus far.

**Approach:** In *chapter 6* we assess the long-term outcome of treatment in dogs with severe DLSS. These dogs were referred to our University Hospital for Companion Animals specifically for this surgery because of failure from a previous decompressive surgery, or as a last resort treatment. In these patients the rigid PSRF construct is used in a permanent way to provide stability and promote spinal fusion. However, this type of construct can also provide temporary distraction of the IVD in patients with early clinical signs of DLSS, with the aim to regenerate the degenerated IVD. Therefore, in *chapter 7* we evaluated the safety and efficacy of temporary IVD distraction by using PSRF in a canine patient with DLSS, in order to regenerate and repair the LS IVD.
References


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Safety of intradiscal injection and biocompatibility of polyester amide (PEA) microspheres in a canine model predisposed to intervertebral disc degeneration

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Abstract

Introduction
Repair of degenerated intervertebral discs (IVD) might be established via intradiscal delivery of biologic therapies.

Methods
Polyester amide polymers (PEA) were evaluated for in vitro cytotoxicity and in vivo biocompatibility, and thereafter intradiscal application of PEA microspheres (PEAMs) in a canine model predisposed to IVD degeneration at long-term (6 months) follow-up.

Results
PEA extracts did not induce cytotoxicity in mouse fibroblast cells (microscopy and XTT assay), while a slight foreign body reaction was demonstrated by histopathology after intramuscular implantation in rabbits. Intradiscal injection of a volume of 40 μl through 26 and 27G needles induced no degenerative changes in a canine model susceptible to IVD disease. Although sham-injected IVDs showed increased CAV1 expression compared with non-injected IVDs, which may indicate increased cell senescence, these findings were not supported by immunohistochemistry, biomolecular analysis of genes related to apoptosis, biochemical and histopathological results. PEAM-injected IVDs showed a significantly higher BAX/BCL2 ratio vs sham-injected IVDs suggestive of an anti-apoptotic effect of the PEAMs. These findings were not supported by other analyses (clinical signs, disc height index, T2 values, biomolecular and biochemical analyses, and IVD histopathology).

Conclusion
PEAs showed a good cytocompatibility and biocompatibility. PEAMs are considered safe sustained release systems for intradiscal delivery of biological treatments.
Intradiscal injection and biocompatibility of PEA microspheres

Introduction
Intervertebral disc (IVD) disease is common in dogs and humans and is associated with IVD degeneration. The process of spontaneous IVD degeneration in chondrodystrophic dogs is similar to that in man, resulting in a valuable animal model for canine and human patients.\textsuperscript{1} Clinical signs, i.e. pain, neurological deficits, develop from an age of 3 – 7 years in these dogs. An aberrant cell-mediated response, associated with genetic predisposition, aging, mechanical overload, and an inadequate metabolite transport results in an dysbalance between anabolic and catabolic processes and consequently a dysfunctional extracellular matrix (ECM).\textsuperscript{2} The nucleus pulposus (NP) and inner annulus fibrosus (AF), normally consisting of mainly collagen type II and large proteoglycan aggregates, change to tissues rich in collagen type I, with a decrease in the size and total amount of proteoglycans, while the fibrils of the outer AF become coarser and more susceptible to injury. These changes ultimately result in disturbance of the structural integrity and biomechanical properties of the IVD.\textsuperscript{2} With increased knowledge on the pathogenesis and biological changes in the diseased IVD, treatment strategies focus on biological repair of the IVD. As the IVD is the largest avascular structure of the body,\textsuperscript{3} direct intradiscal injection via a minimal invasive technique is an elegant way to deliver biological treatments, e.g. cells, growth factors or drugs, into the NP. Growth factors are characterized by short \textit{in vivo} half-lives and chemical instability and to increase their bioavailability \textit{in vivo}, bioactive substances can be encapsulated in biomaterials, e.g. microspheres, hydrogels, that allow sustained release.\textsuperscript{4} In addition, higher loading doses can be achieved locally, without causing systemic side effects, and puncture of the IVD can be reduced to a minimum. A class of polyester amide polymers (PEAs) have gained interest as biodegradable polymers in the past decades, as they possess clear advantages over aliphatic polyester-based biomaterials commonly used.\textsuperscript{5,6} The latter are rather hydrophobic, hydrolytically degradable, and most of them generate acidic products upon degradation. In contrast, PEAs are synthetic, amino-acid-based co-polymers, containing L-amino acids, aliphatic di-carboxylic acids and \(\alpha,\omega\)-diols, creating amphiphilicity, that enhances interactions with proteins and modification with bioactive molecules and (lipophilic) drugs.\textsuperscript{7, 8} Biodegradation of the polymers occurs via surface erosion, and can be accomplished by endogenous enzymes.\textsuperscript{9, 10} PEAs can be manufactured by a polycondensation method and mechanical and thermal properties can be easily tuned.\textsuperscript{8} They have been successfully applied as a coating on coronary stents for sustained drug release,\textsuperscript{11, 12} and were proven to meet the requirements of a biocompatible controlled release system in the ocular environment.\textsuperscript{13, 14} To our knowledge, the intradiscal application of microspheres consisting of PEAs, has not been examined thus far, and seems a promising method to provide sustained release of bioactive substances over a prolonged period in the confined environment of the IVD.
Intradiscal application of therapeutics should be considered with care. In animal models of IVD degeneration, puncture of the IVD induces degeneration, and the gauge size of the needle correlates with the extent of degeneration.\textsuperscript{15-17} Also, a relatively high volume applied intradiscally can cause an increase in hydrostatic pressure, a biomechanical cue shown to induce IVD degeneration.\textsuperscript{18, 19} Furthermore, in human patients, discography injections have been associated with an increased risk of degeneration. As several ongoing clinical trials on intradiscal delivery of biologic therapies are performed (http://clinicaltrials.gov), it is important to assess possible side effects of intradiscal injection to pinpoint the boundary conditions. \textit{In vivo} assessment of safety and efficacy of treatments in clinical trials is obviously limited to radiography and magnetic resonance imaging, which may not be sensitive enough to detect minor changes in the ECM that may have clinically relevant effects on the long term. Assessment of the effect of intradiscal injection at a biochemical, biomolecular, and histopathological level will provide much more information. Preferably this is done in a large animal model with spontaneous IVD degeneration, where underlying pathological processes match human IVD degeneration providing valuable translational information.\textsuperscript{1} In order to assess the effects of intradiscal injection of a sham condition in IVDs in dogs predisposed to IVD degeneration, we evaluated magnetic resonance imaging (MRI), in combination with biochemical, biomolecular and histopathological data at long-term follow-up (6 months). Furthermore, after evaluating the biocompatibility and safety of intramuscular application of PEAs in rabbits, we also evaluated the long-term effects on IVD integrity of PEA microspheres (PEAMs) after intradiscal application (6 months).

\textbf{Materials and methods}

\textbf{Ethics statement}

All procedures involving animals were approved and conducted according to US regulation (rabbits), and to Dutch regulation (dogs; experimental number: 2012.III.07.065).

\textbf{Synthesis of polyester amide polymers}

PEAs were synthesized according to a previously published method.\textsuperscript{13, 20} Briefly, the polymer was prepared via polycondensation of 0.45 equivalents of di-p-toluenesulfonyl acid salts of bis-(L-leucine)\textsubscript{1},4-dianhydroosorbitol diester (1), 0.30 equivalents of bis-(L-leucine) \textsubscript{α,ω}-hexanediol diester (2), 0.25 equivalents of lysine benzyl ester (3), and 1 equivalent of di-N-hydroxysuccinimide ester of sebacic acid (4) in anhydrous dimethylformamide and trimethylamine in a glass vessel with overhead stirrer under a nitrogen atmosphere. Employing pre-activated acid in the reaction allows polymerization at a relative low temperature (65 °C), resulting in a polycondensate free of by-products and predictable degradation components. The polymer with an average number molecular weight of 48 kDa was isolated from the reaction mixture in two precipitation
Preparation of PEA microspheres (PEAMs)

A volume of 30 ml of 1% filtered polyvinyl alcohol (PVA) was stirred with an Ultra-Turrax® (IKA Labortechnik, Staufen, Germany) equipped with a 25S – 10G stirring rod) at 4000 rpm. A volume of 150 μl of 10% trehalose in water was added to 8.5% polymer in dichloromethane (DCM) and vortexed for 30s at 13000 rpm. This emulsion was injected into the PVA solution and stirred at 4000 rpm. After 3 minutes the Ultra-Turrax was removed, a stirring bar was added and the emulsion was stirred for another 16 hours. After stirring, the PEAMs were allowed to sink to the bottom of the vial and the supernatant was removed. The PEAMs were washed three times with 20 ml of 0.04% ice-cold, filtered Tween® 20. Following the final washing about 5 ml of 0.04% Tween® 20 was added, and the PEAMs were lyophilized. The average diameter of the microspheres was 40 μm.

In vitro cytotoxicity polyester amide polymers

For cytotoxicity testing, mouse L-929 fibroblast cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Lonza), 1% penicillin/streptomycin (pen/strep), and plated into 96-well plates at a density of 9 x 10^3 cells/cm². Autoclaved natural rubber and silicone served as a positive and a negative control, respectively. Extractions of 4 g (surface ± 60 cm²) of gamma-irradiated polymer specimens were prepared in 20 ml cell culture medium (MEM Eagle with Earle’s BSS with L-glutamine, Lonza) 10% FBS + 1% pen/strep and subsequently incubated at 37 °C for 24 h in a humidified atmosphere (5% CO₂). Cells were exposed to the PEA extracts or controls at 37 °C for 48 h (5% CO₂). Cell viability was immediately recorded by microscopic examination of the cells and by using the XTT assay. Cell viability assays were performed with three or more replicates.

Biocompatibility of polyester amide polymers in rabbits

Three healthy adult New Zealand White rabbits (Charles River, Wilmington, MA, USA) were fully anesthetized and six sterilized strips of PEAs of 1 x 1 x 10 mm in size were implanted into the paravertebral muscles under sterile conditions. Six strips of plastic served as a control. Rabbits were monitored daily for signs of distress or pain (e.g. lethargy, weight loss, automutilation, and abnormal posture) and injection sites were monitored for inflammation (e.g. swelling, redness, pain, and heat). After 2 weeks all animals were sacrificed. The implants were excised, macroscopically evaluated and fixed in 4% neutral buffered formaldehyde. Paraffin sections of 4 μm were stained with hematoxylin and eosin and histopathologically assessed for infiltration of inflammatory
cells, giant cells, necrosis, neovascularization, fatty infiltration, and the encapsulation of the biomaterial by a fibrotic capsule.

**Long term (6-month) effect of intradiscal injection on canine intervertebral discs**

This study was performed in seven intact male beagle dogs (Harlan) with a median age of 1.3 years (range 1.1 – 1.8) and a median weight of 11.7 kg (range 10.2 – 12.8). A board-certified veterinary surgeon (BM) performed a general, orthopedic, and neurologic examination on all dogs. A blood sample was drawn from the jugular vein to assess white blood cell count and differentiation, to exclude systemic inflammation. T2-weighted images were obtained pre- (t₀) and post-operatively at 6 (t₆), 12 (t₁₂), and 24 (t₂₄) weeks under general anesthesia using a 1.5 Tesla scanner (Philips Healthcare, Best, The Netherlands). All lumbar IVDs were assessed at all 4 time points according to the Pfirrmann score, by a veterinary radiologist that was blinded to treatment allocation on sagittal T2-weighted images. Only lumbar IVDs with a Pfirrmann score II were included in the study. All injections were performed by the same person (BM), and non-injected IVDs served as controls. Briefly, T₁₂ – L₅ were exposed and injected via a left lateral approach, and L₆ – S₁ via a dorsal approach. A 100 µl gastight syringe (7656-01 Model 1710 RN) was used to inject 40 µl of a sham containing 1% sucrose, 1.2% mannitol, 20 mM glycine, and 0.05% Tween® 20 through a 27G needle (25 mm, 12° beveled point; Hamilton Company USA, Reno, Nevada, USA). A volume of 40 µl of 1.3% PEAMs in 0.9% NaCl needed to be injected through a 26G needle (25 mm, 12° beveled point; Hamilton) to avoid clogging by the microspheres (unpublished data). The location of the tip of the needle in the NP was estimated by the length of passage through the AF (1 cm), while constant resistance was encountered. Once the NP was encountered, resistance decreased and the volume could be easily injected. The needle was slowly pulled back to allow the AF puncture site to close, and the site was inspected for extrusion of the administered substance.

Disc height index (DHI) was calculated at all 4 time points on T2-weighted images. At all 4 time points, quantitative T2 maps were generated from a multi-echo imaging sequence with 8 echoes. For the analysis of T2 values in the NP, an oval-shaped region of interest (ROI) in the NP was manually placed on mid-sagittal IVD sections. ROIs were exported to, and analyzed with Wolfram Mathematica 10.0 (Wolfram Research, Champaign, IL, USA). T2 values were computed by calculating the mean signal intensity (S) in each ROI, and by fitting these intensity data to the following equation: \( S(TE) = S₀ e^{-TE/T₂} \), using the Levenberg-Marquardt nonlinear least-squares method implemented in Mathematica. \( S₀ \) denotes the equilibrium magnetization, whereas \( S(TE) \) indicates the signal as a function of echo time (TE).
Sample collection, macroscopic grading, and histopathological grading of canine intervertebral discs

Dogs were euthanized 6 months after intradiscal injection by way of sedation with dexmedetomidine followed by pentobarbital. The vertebral column (T12 – S1) was harvested to generate nine spinal units. One part of the IVD tissue, containing both NP and AF, was snap frozen in liquid nitrogen and stored at -80°C for biochemical and biomolecular analyses. The other part was photographed (Olympus VR-340, Hamburg, Germany) for macroscopic evaluation, and fixed in 4% buffered formaldehyde at 4°C for 2 weeks. Two independent investigators, blinded to the treatments, evaluated macroscopic images of the IVD segments according to the Thompson grading scheme. Samples were decalcified according to Kristensen in 35% formic acid and 6.8% sodium formate. Paraffin sections (5 μm) stained with hematoxylin/eosin and with picrosirius red/alcian blue were histopathologically evaluated by two independent investigators, blinded to the treatments, according to the grading scheme developed by Bergknut et al. Immunohistochemistry for caveolin-1 (monoclonal mouse anti-caveolin-1 antibody (Clone 2297, 610406, BD Biosciences) diluted 1:50 in PBS) was performed as described previously.

RNA isolation and quantitative polymerase chain reaction of the nucleus pulposus and the annulus fibrosus

Transverse cryosections (60 μm) of the IVDs were collected and the NP and AF tissues were separated visually. One half was collected in 300μl RLT buffer containing 1% β-mercapto-ethanol (Qiagen, Venlo, The Netherlands) and stored at -80°C until biomolecular analyses. Total RNA was isolated from the NP and AF tissues by using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. The incubation period with proteinase K was reduced to five minutes increase RNA yield. After on-column DNase-I digestion (Qiagen RNase-free DNase kit), RNA was quantified by using a NanoDrop 1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). The iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) was used to synthesize cDNA. Quantitative PCR (qPCR) was performed using an iCycler CFX384 Touch termal cycler, and IQ SYBRGreen Super mix (Bio-Rad)to assess the effects at gene expression levels with regards to: 1) ECM anabolism: aggrecan (ACAN), collagen type II (COL2A1), collagen type I (COL1A1); 2) ECM catabolism: a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS5), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinase 1 (TIMP1); 3) proliferation: cyclin-D1 (CCND1) and 4) apoptosis: caveolin-1 (CAV1), caspase 3 (CASP3), and B-cell lymphoma 2-associated X/B-cell lymphoma 2 (BAX/BCL2) ratio (Additional file 1). Relative expression levels were determined by normalizing the C_t value of each target gene by the mean C_t value of 4 reference genes, i.e. glyceraldehyde 3-phosphate...
dehydrogenase (GAPDH), ribosomal protein S18 (RPS19), succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA), and hypoxanthine-guanine phosphoribosyltransferase (HPRT).

**Biochemical assays: glycosaminoglycan, collagen and DNA**

The other half of the cryosectioned NP and AF tissues was digested overnight in papain buffer (250 μg/ml papain (P3125-100 mg, Sigma-Aldrich) + 1.57 mg/ml cysteine HCL (C7880, Sigma-Aldrich)) at 60°C. Glycosaminoglycan (GAG) content was quantified by using a 1,9-dimethylmethylene blue assay. The Quant-iT™ dsDNA Broad-Range assay kit in combination with a Qubit™ fluorometer (Invitrogen, Carlsbad, USA) was used in accordance with the manufacturer’s instructions. Collagen was quantified by using a hydroxyproline assay and calculated from the hydroxyproline content by multiplying with a factor 7.5. Total GAG and collagen content were normalized for DNA content of the NP and AF.

**Statistical analysis**

All data were analyzed by using R statistical software, package 3.0.2. Residual plots and quantile-quantile (Q-Q)-plots were used to check normality of the data. In case of violation of the assumption of normality, data were logarithmically transformed. A linear mixed effect model was used to analyze the effect of intradiscal injection and intradiscal application of PEAMs on DHI, T2 values, histopathological grading, GAG, collagen and DNA content. Model selection was based on the lowest Akaike Information Criterion (AIC). The correlation between multiple measurements within one dog was taken into account by incorporating ‘dog’ (dog 1 – 7) as a random effect. DHI and T2 values were corrected for t₀, and ‘treatment’, ‘time’ (t₆, t₁₂, and t₂₄), and their interaction served as fixed effect factors. ‘Treatment’ (non-injected, sham, PEAMs) served as a fixed factor in the analysis of histopathological scores. For the analysis of GAG and DNA levels, ‘treatment’, ‘tissue’ (NP and AF), and their interaction were incorporated into the model as fixed effect factors. The Cox proportional hazards regression model was used to estimate the effect of the injected treatments on gene expression levels. Calculations were performed on the ratio of the Cₜ values for each target gene to the mean Cₜ value of the reference genes. Cₜ values ≥ 40 were right censored. Regression coefficients were estimated by the maximum likelihood method. Differences between treatments were considered significant if 0 was not included in the confidence interval, whereas hazard ratios were considered significant if 1 was not included in the confidence interval. Confidence intervals were calculated and stated at the 99% confidence level to correct for multiple comparisons.
Results

Cytocompatibility of PEA in vitro and biocompatibility of polyester amide polymers and polyester amide microspheres in vivo

No visible signs of toxicity in response to the PEA were observed in the fibroblast monolayers at microscopic evaluation. Cell viability, as measured by the XTT assay, was 97.9% after 48 h incubation with PEA dispersions, and was considered non cytotoxic.\textsuperscript{29} Macroscopic evaluation of the PEA implant sites in rabbits indicated no significant signs of inflammation, encapsulation, hemorrhage, necrosis, or discoloration after two weeks. Microscopic evaluation of the implant sites indicated no significant signs of fibrosis, hemorrhage, necrosis, or degeneration compared with the control implant sites. Cellular infiltrates and giant cells were seen at the interface of the test implant sites. Individual scores of the rabbits are shown in Additional file 2. Overall, implantation of PEA specimens demonstrated a slight foreign body reaction intramuscularly.

Intradiscal injection in a canine model of spontaneous intervertebral disc degeneration

All dogs recovered from surgery uneventfully. All 21 injected IVDs were scored a Pfirrmann grade II before surgery (t\textsubscript{0}). Pfirrmann scores of 20/21 IVDs and T2-values of all IVDs were not significantly different over time (t\textsubscript{6}, t\textsubscript{12}, t\textsubscript{24}). T2-values per condition are described in Additional file 3. One (1/21) sham-injected IVD was scored a Pfirrmann grade III at all subsequent time points. The mean DHI of non-injected, sham-injected, and PEAMs-injected IVDs were not significantly different (Figure 1a). Mean disc height of non-injected IVDs was 3.84 mm (range 3.20 – 4.93 mm). As the outer diameter of a 27G needle is 0.41 mm and of a 26G needle 0.47 mm, the ratios of needle diameter to disc height were calculated at 11% and 12% respectively. The volume injected in this study consisted of 20% of the total NP volume (i.e. 40 μl of 200 mm\textsuperscript{3}).\textsuperscript{30} Post-mortem, all IVDs were scored a Thompson grade II, in accordance with early IVD degeneration. Representative macroscopical images of all three conditions are shown in Figure 1B, 1C, and 1D.

Histopathology and biochemistry

The median histopathological grade of non-injected (12; range: 10 – 15), sham-injected (14; range: 11 – 17), and PEAMs-injected IVDs 13 (11 – 15) was not significantly different. Representative histopathological images (picrosirius red/alcian blue stain) of all conditions are shown in Figure 1B', 1B'', 1C', 1C'', and 1D', 1D''. In one of the PEAMs-injected IVDs (level L7 – S1) a small pyogranulomatous reaction in the dorsal ligament and outer layer of the dorsal AF was detected, indicative of an inflammatory response. GAG and collagen levels corrected for DNA were not different between conditions (Figure 2A and 2B). In all conditions, GAG/DNA was significantly higher in the NP compared with the AF (M 0.55, SD 0.04; CI99% 0.44 – 0.66).
Figure 1. A. Change in disc height expressed as the percent disc height index compared with the pre-injection value (set at 100%) in non-, sham, and polyester amide polymer microspheres (PEAMs)-, injected canine IVDs at 6 months follow-up ± standard deviation. B – D. Representative macroscopical (B – D) and histopathological images (picrosirius red/alcian blue stain) of non-injected (B’– B’’), intradiscally injected (sham (C’– C’’)) and PEAMs (PEAMs (D’– D’’)) canine IVDs at 6 months follow-up. B’’, C’’, D’’ are magnifications of the squares in B’, C’, D’, respectively. B – D. Nuclei pulposi (NPs) in all conditions had a bulging aspect (asterisk) due to the processing method and the tissue properties. Regardless of the treatment, at macroscopy a white opaque NP could be noticed in all IVDs, consistent with early IVD degeneration. Histopathologically, also regardless of the treatment, small size chondrocyte groups, consisting of 2 – 7 cells (arrow), within a mixture of collagen-rich (red stain) and glycosaminoglycans-rich (GAG; blue and green stain) extracellular matrix were observed in the NP of all IVDs.
Figure 2A-G. Relative gene expression levels in non-, sham-, and polyester amide microspheres (PEAM)-injected, canine intervertebral discs (IVDs) at 6 months follow-up. The non-injected (Non-inj) nuclei pulposi (NP) are set at 1. A, B, and C Gene expression levels of collagen type II alpha 1 (COL2A1) (a) and collagen type II alpha 1 (COL1A1) (B) were not significantly different between conditions, neither was collagen corrected for DNA (C). D. Caspase 3 (CASP3) expression levels were significantly lower in the annulus fibrosus (AF) compared with the NP. E. Expression levels of caveolin-1 (CAV1) were significantly higher in sham-injected IVDs (NP + AF) compared with non-injected control IVDs. F. In all conditions glycosaminoglycan (GAG) corrected for DNA were significantly higher in the NP compared with the AF. G. The PEAM-injected IVDs showed a significant higher B-cell lymphoma 2-associated X/B-cell lymphoma 2 (BAX/BCL2) ratio compared with the sham-injected IVDs. Data in A, B, D, and E are expressed as n-fold changes, in C and F as mean values, and in G as ratio ± standard deviation.* Indicates significant difference at a 99% confidence interval.
Gene expression apoptotic and degenerative pathways and immunohistochemistry

Relative gene expression of \textit{CASP3} in the AF was significantly lower (HR 0.21; CI99\% 0.07 – 0.66) than in the NP in all conditions and could be suggestive of a higher apoptotic rate in the NP (Figure 2C). The \textit{BAX/BCL2} ratio in the sham-injected IVDs (NP and AF) was significantly lower (HR 0.14; CI99\% 0.02 – 0.85) compared with the PEAMs-injected IVDs, indicative of an anti-apoptotic effect of the PEAMs (Figure 2D). In addition, \textit{CAV1} gene expression was significantly higher (HR 3.30; CI99\% 1.00 – 10.83) in sham-injected IVDs (NP and AF) compared with non-injected IVDs (Figure 2E). None of the IVDs showed staining of cells with anti-caveolin-1 antibody in the NP and/or AF. Relative gene expression of anabolic (\textit{ACAN, COL2A1} (Figure 2F), \textit{COL1A1} (Figure 2G)), catabolic (\textit{MMP13}), anti-catabolic (\textit{TIMP1}), and proliferative (\textit{CCND1}) genes were not significantly different (Additional file 4). Gene expression of \textit{ADAMTS5} was below detectable levels in the NP as well as the AF in all conditions.

Discussion

In this study, intradiscal injection of a volume of 40 \(\mu\)l through 26 and 27G needles induced no degenerative changes in a canine model predisposed to IVD degeneration at long-term (6 months) follow-up. Although sham-injected IVDs showed increased \textit{CAV1} expression compared with non-injected IVDs, which may indicate increased cell senescence, these findings were not supported by immunohistochemistry, biomolecular analysis of genes related to apoptosis, biochemical and histopathological results.

A needle puncture has been described to alter mechanical properties by reducing pressure in the NP and/or damaging the AF, depending on the diameter of the needle. The rabbit annular stab model of induced IVD degeneration is based upon this concept. \textit{IVD} degeneration has been observed when the ratio of needle diameter to disc height exceeded 40\%. With regard to injection volume, the NP can be considered as a confined space in which the hydrostatic pressure will increase if a substance is injected. In the caudal IVDs of rats, volume-dependent degenerative changes have been demonstrated after injection of phosphate buffered saline at a radiographic, biochemical, and histopathological level. Although the exact threshold volumes were not specified, injected volumes up to 66\% (i.e. 2 \(\mu\)l of 3.1 mm\(^3\)) of the total NP volume, showed no degenerative changes at short-term follow-up. Needle size diameters and injected volume applied in our study were well within the safety ranges described in literature, and induced no degenerative changes in canine IVDs predisposed to degeneration.

Furthermore, we showed a good cytocompatibility \textit{in vitro} and biocompatibility of PEAs in rabbits. Only a slight foreign body reaction was seen after intramuscular implantation in rabbits. We hypothesized that intradiscal application of PEAs in the avascular IVD would
Intradiscal injection and biocompatibility of PEA microspheres

be well accepted, in line with previous studies showing a moderate subcutaneous tissue response to a hydrogel that showed no response at all upon intradiscal injection.\textsuperscript{33} Furthermore, safe intravitreal application of PEA fibrils through a 26G needle was already shown by Kropp et al\textsuperscript{13}. Indeed, PEAMs were considered safe within a 6 months follow-up period, based on clinical signs, disc height index, T2 values, biomolecular and biochemical analyses, and IVD histopathology. The focal, mild granulomatous reaction described in one of the PEAM injected IVDs could have been a consequence of leakage of the PEAMs, the injection procedure itself, or it could have been a reaction consistent with the ongoing process of IVD degeneration.\textsuperscript{34} The PEAMs-injected IVDs showed a significant higher $BAX/BCL2$ ratio compared with the sham-injected IVDs, suggestive of an anti-apoptotic effect of the PEAMs. However, neither substance significantly affected this parameter compared with the non-injected controls, thus questioning the implications of this finding.

Several clinical trials are currently being performed, in which promising regenerative treatments, consisting of cell, gene and protein therapies, are injected into the IVD (http://clinicaltrials.gov). In order to use PEAMs as a part of a regenerative therapy, effectuating sustained release of bioactive substances in the degenerative IVD, release profiles and degradation processes in the intradiscal environment need to be investigated in future. The stage of degeneration has been shown to be an important factor in the efficacy of treatment with cell-based therapies, which emphasizes the importance of the preclinical canine model that resembles the human situation.\textsuperscript{35}

**Conclusion**

In conclusion, we showed a good cytocompatibility *in vitro* and biocompatibility of PEAs in rabbits. Intradiscal injection of 40 $\mu$l of a sham condition through a 27G needle, and of PEA microspheres through a 26G, could be safely applied in a large animal model predisposed to IVD degeneration without accelerating degeneration over the course of 6 months.

**Acknowledgements**

This research forms part of the Project P2.01 IDiDAS of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. The financial contribution of the Dutch Arthritis Foundation is gratefully acknowledged (IDiDAS, LLP22 and LLP12). We would like to acknowledge Toxikon Corporation (Bedford, MA, USA) for performing the cytocompatibility tests *in vitro*, and the biocompatibility tests *in vivo*. Furthermore we would like to express our sincere appreciation to Saskia Plomp and Jeannette Wolfsinkel for their assistance in the laboratory.
References


**Table 1.** Primers used for quantitative polymerase chain reaction (qPCR)

<table>
<thead>
<tr>
<th>Reference genes</th>
<th>Protein</th>
<th>Forward sequence 5’ -&gt; 3’</th>
<th>EXON</th>
<th>Reverse sequence 5’ -&gt; 3’</th>
<th>EXON</th>
<th>Amp. size</th>
<th>Ann. temp (°C)</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>TGTCCCCACCCCCCAATGTATC</td>
<td>7</td>
<td>CTCCGATGCTGCTTCCTACCTTT</td>
<td>8</td>
<td>100</td>
<td>58</td>
<td>NM_001003142</td>
<td></td>
</tr>
<tr>
<td><strong>HPRT</strong></td>
<td>AGCTTGCTGGTGAAAAGGAC</td>
<td>5/6</td>
<td>TTATAGTCAAGGGGATATCC</td>
<td>7</td>
<td>104</td>
<td>58</td>
<td>NM_001003357</td>
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</tr>
<tr>
<td><strong>RPS19</strong></td>
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<td>2/3</td>
<td>GTTCTCATCTAGGGAGGCAAG</td>
<td>3</td>
<td>95</td>
<td>61</td>
<td>XM_005616513</td>
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<tr>
<td><strong>SDHA</strong></td>
<td>GCCCTGGATCTCTTGTATGGA</td>
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<td>TTCTTGGCTTCTATGCTAGTG</td>
<td>6</td>
<td>92</td>
<td>61</td>
<td>DQ_402985</td>
<td></td>
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<td><strong>Target genes</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACAN</strong></td>
<td>GGACACTCCTTGCAATTGAG</td>
<td>13/14</td>
<td>GTCACTCCACTCTCCCTTCT</td>
<td>14</td>
<td>111</td>
<td>62</td>
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<td></td>
</tr>
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<td><strong>ADAMTS5</strong></td>
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<td>GAACCATCACAAGGATGTC</td>
<td>6</td>
<td>149</td>
<td>61</td>
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</tr>
<tr>
<td><strong>BAX</strong></td>
<td>CCTTTGTCTCTAGGGTCCA</td>
<td>2/3</td>
<td>CTCAGCTTCTTGGTGATGC</td>
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<td>108</td>
<td>59</td>
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<tr>
<td><strong>BCL2</strong></td>
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<td>AGGTTGTCAGATGCGGCTAGGT</td>
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<td>62</td>
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<tr>
<td><strong>CASP3</strong></td>
<td>ATCACAAGTAGGATGGGGGTTGGT</td>
<td>8</td>
<td>TGAAGGAGCATGGTCTGAAGTAGCAGTC</td>
<td>8</td>
<td>139</td>
<td>58</td>
<td>NM_001003042</td>
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<tr>
<td><strong>CAV1</strong></td>
<td>GGCACACCAAGGAATACTG</td>
<td>2</td>
<td>AAATCAATCTTTGACCAACGTC</td>
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<td>60</td>
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<tr>
<td><strong>CCND1</strong></td>
<td>GGCTGGAAGATGAAGGAG</td>
<td>1</td>
<td>CAGTTTGTCTCAACAGGAGCA</td>
<td>1</td>
<td>151</td>
<td>60</td>
<td>NM_001005757</td>
<td></td>
</tr>
<tr>
<td><strong>COL1A1</strong></td>
<td>GGTGTGCTACAGGAAGGGCCCTCA</td>
<td>2</td>
<td>TCACCGACTCAAGCTAGTC</td>
<td>2</td>
<td>109</td>
<td>61</td>
<td>NM_001003090</td>
<td></td>
</tr>
<tr>
<td><strong>COL2A1</strong></td>
<td>GCCAGAAAAAGAGGAG</td>
<td>52</td>
<td>TTCTGAGAGCCTGGT</td>
<td>53</td>
<td>151</td>
<td>62</td>
<td>XM_0056366764</td>
<td></td>
</tr>
<tr>
<td><strong>MMP13</strong></td>
<td>CTGAGGAGACTTCTGAGCT</td>
<td>2</td>
<td>TTGGACACTTTGAGAGTTC</td>
<td>2</td>
<td>250</td>
<td>65</td>
<td>XM_536598</td>
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<tr>
<td><strong>TIMP1</strong></td>
<td>GGCGTTATGAGTAGTAAGATGAC</td>
<td>2</td>
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<td>3</td>
<td>120</td>
<td>66</td>
<td>NM_001003182</td>
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</tr>
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</table>

Primer sets for qPCR analysis of reference genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT), ribosomal protein S19 (RP19), succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA), and target genes: aggrecan (ACAN), a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS5), B-cell lymphoma 2-associated X (BAX), B-cell lymphoma 2 (BCL2), caspase 3 (CASP3), caveolin-1 (CAV1), cyclin-D1 (CCND1), collagen type I (COL1A1), collagen type II (COL2A1), matrix metalloproteinase 13 (MMP13), and tissue inhibitor of metalloproteinase 1 (TIMP1). Amp. = amplicon, ann. = annealing
Additional file 2. Histopathological evaluation of intramuscularly implanted polyester amide polymers in rabbits

Implantation tests were performed to evaluate the local effects of implanted test articles on living tissue, at both macroscopic and microscopic level of test articles that were surgically implanted into an appropriate implant site. Hence, test and control materials were implanted into the paravertebral muscle of each of three rabbits. At the end of the observation period of 2 weeks, the area of the tissue surrounding the centre position of each implant strip was examined macroscopically. Subsequently, the implanted sites were processed for histopathologic evaluation by a veterinary pathologist. Inflammation, fibrosis, hemorrhage and necrosis were evaluated on a scale and compared to the control article sites. Polyester amide polymers (PEA) and control implant sites were processed for histopathological evaluation by a veterinary pathologist for each animal using tables 1 and 2.

Table 1. Histological evaluation of inflammatory responses.

<table>
<thead>
<tr>
<th>Cell type/response</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Polymorphonuclear cells</td>
<td>Rare, 1-5/phf</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Rare, 1-5/phf</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>Rare, 1-5/phf</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Rare, 1-5/phf</td>
</tr>
<tr>
<td>Giant cells</td>
<td>Rare, 1-5/phf</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Minimal</td>
</tr>
</tbody>
</table>

Phf = per high powered field (x 400)

Table 2. Histological evaluation of healing responses.

<table>
<thead>
<tr>
<th>Cell type/response</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Neovascularisation</td>
<td>Minimal capillary, proliferation, focal, 1-3 buds</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Narrow band</td>
</tr>
<tr>
<td>Fatty Infiltrate</td>
<td>Minimal amount of fat associated with fibrosis</td>
</tr>
</tbody>
</table>

Phf = per high powered field (x 400)
The relative size of the involved area was scored by assessing the width of the area from the implant/tissue interface to unaffected areas which have the characteristics of normal tissue and normal vascularity. Relative size of the involved are was scored using the following scale:

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0 mm</td>
</tr>
<tr>
<td>1</td>
<td>up to 0.5 mm</td>
</tr>
<tr>
<td>2</td>
<td>0.6 – 1.0 mm</td>
</tr>
<tr>
<td>3</td>
<td>1.1 – 2.0 mm</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 2.0 mm</td>
</tr>
</tbody>
</table>

For each implanted site, a total score was determined. The inflammatory response was added up and weighted by a factor of two (2). The healing responses were added up separately. Thereafter, the total score of the inflammatory and healing responses was calculated for each site.

The separate tables of the histopathological evaluation of each tested implant and respective control sites 4 weeks after intra-muscular implantation in 3 New Zealand White rabbits are presented below:

<table>
<thead>
<tr>
<th>Categories of reaction</th>
<th>Test sites</th>
<th>Control sites</th>
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</thead>
<tbody>
<tr>
<td>Animal #1</td>
<td>T1 T2 T3 T4 T5 T6 C1 C2 C3 C4 C5 C6</td>
<td></td>
</tr>
<tr>
<td>Foreign debris</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Rel. size</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1</td>
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<tr>
<td>Polymorphonuclear cells</td>
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</tr>
<tr>
<td>Lymphocytes</td>
<td>1 0 0 0 1 1 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>2 1 2 1 1 2 1 1 1 1 1 1</td>
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</tr>
<tr>
<td>Giant cells</td>
<td>0 0 1 1 0 1 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
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</tr>
<tr>
<td>Subtotal (x2)</td>
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</tr>
<tr>
<td>Neovascularization</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Fatty infiltrate</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Subtotal (x1)</td>
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<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>12 6 12 8 10 14 6 6 6 6 6 6</td>
<td></td>
</tr>
<tr>
<td>Categories of reaction</td>
<td>Test sites</td>
<td>Control sites</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Animal #2</td>
<td>T1 T2 T3 T4 T5 T6 C1 C2 C3 C4 C5 C6</td>
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</tr>
<tr>
<td>Foreign debris</td>
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<td></td>
</tr>
<tr>
<td>Rel. size</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1</td>
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<tr>
<td>Polymorphonuclear cells</td>
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<tr>
<td>Lymphocytes</td>
<td>0 0 0 0 0 1 0 0 0 0 0 0</td>
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</tr>
<tr>
<td>Plasma cells</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
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</tr>
<tr>
<td>Macrophages</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1</td>
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<tr>
<td>Giant cells</td>
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<tr>
<td>Necrosis</td>
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<td><strong>Subtotal (x2)</strong></td>
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<tr>
<td>Neovascularization</td>
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<tr>
<td>Fibrosis</td>
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<tr>
<td>Fatty infiltrate</td>
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</tr>
<tr>
<td><strong>Subtotal (x1)</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>10 10 10 10 14 NA 6 6 6 6 6 6</td>
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<table>
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<th>Categories of reaction</th>
<th>Test sites</th>
<th>Control sites</th>
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<tr>
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<tr>
<td><strong>Subtotal (x1)</strong></td>
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<td></td>
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<tr>
<td><strong>TOTAL</strong></td>
<td>10 10 10 8 12 10 NA 6 6 8 8 6</td>
<td></td>
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</tbody>
</table>

NA: not available; * the PEA in situ was not scored as foreign debris
Based on the average score of the test and control sites, the average difference between test and controls for each animal was calculated.

<table>
<thead>
<tr>
<th>Animal #</th>
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<th>control</th>
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<tr>
<td>Mean</td>
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Based on ISO10993-6, 2007, Biological Evaluation of Biomedical Devices – part 6, biocompatibility was rated at 4,1 compatible with a slight reaction based on the following scheme:

- 0.0 – 2.9: No reaction
- 3.0 – 8.9: Slight reaction
- 9.0 –15.0: Moderate reaction
- > 15: Severe reaction
Additional file 3. T2-values per condition

T2-values in canine non-, sham-, and polyester amide polymer microspheres (PEAM)- injected intervertebral discs showed no significant changes at 6, 12, and 24 weeks.

Additional file 4. Relative gene expression levels in canine intervertebral discs at 6 months follow-up.

Relative gene expression levels in canine non-, sham-, and PEAMs- injected canine IVDs at 6 months follow-up. The non-injected (Non-inj) nuclei pulposi (NP) are set at 1. Gene expression levels of aggrecan (ACAN) (a), matrix metalloproteinase 13 (MMP13) (b), tissue inhibitor of metalloproteinase 1 (TIMP1) (c), and cyclin-D1 (CCND1) (d) were not significantly different between conditions. Data are expressed as n-fold changes ± the standard deviation.
Intradiscal application of rhBMP-7 does not induce regeneration in a canine model of spontaneous intervertebral disc degeneration

Nicole Willems, Frances C. Bach, Saskia G.M. Plomp, Mattie H.P. van Rijen, Jeannette Wolswinkel, Guy C.M. Grinwis, Clemens Bos, Gustav J. Strijkers, Wouter J.A. Dhert, Björn P. Meij, Laura B. Creemers, Marianna A. Tryfonidou

Arthritis Research & Therapy (2015) 17:137
Abstract

Introduction
Strategies for biological repair and regeneration of the intervertebral disc (IVD) by cell and tissue engineering are promising, but few have made it into the clinic. Recombinant human bone morphogenetic protein 7 (rhBMP-7) has been shown to stimulate matrix production by IVD cells in vitro, and in vivo in animal models of induced IVD degeneration. The aim of this study was to determine the most effective dose of intradiscally injected rhBMP-7 in a spontaneous canine IVD degeneration model for translation into a clinical application for patients with low back pain.

Methods
Canine nucleus pulposus cells (NPCs) were cultured with rhBMP-7 to assess the anabolic effect of rhBMP-7 in vitro, and samples were evaluated for glycosaminoglycan (GAG) and DNA content, histology, and matrix-related gene expression. Three different dosages of rhBMP-7 (2.5 μg, 25 μg, and 250 μg) were injected in vivo into early degenerated IVDs of canines, which were followed up for 6 months by magnetic resonance imaging (T2-weighted images, T1p and T2 maps). Post-mortem, the effects of rhBMP7 were determined by radiography, computed tomography and macroscopy, and by histological, biochemical (GAG, DNA, collagen), and biomolecular analyses of IVD tissue.

Results
In vitro, rhBMP-7 stimulated matrix production of canine NPCs as GAG deposition was enhanced, DNA content was maintained and gene expression levels of ACAN and COL2A1 were significantly upregulated. Despite the wide dose range of rhBMP7 (2.5 – 250 μg) administered in vivo, no regenerative effects were observed at the IVD level. Instead, extensive extradiscal bone formation was noticed after intradiscal injection of 25 μg and 250 μg of rhBMP-7.

Conclusion
Intradiscal bolus injection of 2.5, 25, and 250 μg rhBMP-7 showed no regenerative effects in a spontaneous canine IVD degeneration model. In contrast, intradiscal injection of 250 μg rhBMP-7, and to a lesser extent 25 μg rhBMP-7, resulted in extensive extradiscal bone formation, indicating that a bolus injection of rhBMP-7 alone cannot be used for treatment of IVD degeneration in human or canine patients.
Intradiscal application of rhBMP-7 | 67

Introduction

Low back pain is one of the major sources of disability in humans. Several studies have provided evidence for its association with intervertebral disc (IVD) degeneration. Current therapies, such as physiotherapy, anti-inflammatory medications, and surgery alleviate symptoms, but do not restore the physiological function of the degenerated IVD. Prevention of further degeneration or regeneration of the IVD require intervention at an early stage. Strategies for biological repair and regeneration of the IVD by cell and tissue engineering are promising, but are not widely clinically applicable thus far. A number of studies have been performed on bone morphogenetic proteins (BMPs) given their potential regenerative role in degenerative IVD disease. BMPs belong to the transforming growth factor-β (TGF-β) superfamily, and are involved in many developmental processes. Recombinant human bone morphogenetic protein 7 (rhBMP-7) has been tested extensively and appears to be a promising BMP for IVD regeneration, as it has been shown to have beneficial effects on extracellular matrix production of rabbit, bovine, and human IVD cells in vitro.

Several animal models with experimental IVD degeneration were used to study the efficacy and translational aspects of BMP-7 towards a clinical application in humans. In rabbits with induced IVD degeneration, rhBMP-7 restored disc height and improved the IVD viscoelastic properties by increasing the proteoglycan content. An anti-catabolic effect of rhBMP-7 was shown in a rat model with induced IVD degeneration. Also in a canine model of allogenic IVD transplantation, nucleus pulposus cells (NPCs) expressing rhBMP-7, prevented degeneration of the transplanted IVD at 6 months follow-up. Thus far, novel regenerative therapies deploying rhBMP-7 intradiscally have been tested in animal models with induced IVD degeneration, but not in an animal model with spontaneous IVD degeneration that more closely resembles the biological condition in humans. Furthermore, dose response studies evaluating intradiscal injection of rhBMP-7 and possible adverse effects are not available.

The goal of this study was to assess the effect of a wide range of intradiscally injected dosages of rhBMP-7 (2.5 – 250 µg) in a canine model with spontaneous IVD degeneration that closely resembles IVD degeneration and disease in man. For this, we first investigated the anabolic effect of two dosages of rhBMP-7 on early degenerated canine NPCs in vitro. Potential regenerative effects of rhBMP-7 in vivo were studied by obtaining conventional T2-weighted images and T2 and T1ρ maps in a longitudinal manner. Both T1ρ and T2 relaxation times are correlated with IVD degeneration since T2 relaxation times correlate strongly with water content, while T1ρ relaxation times are particularly sensitive to a decrease in glycosaminoglycan (GAG) content in the NP. At 6 months follow up, the effects of rhBMP7 were determined post-mortem by radiography and computed tomography, macroscopy, and by histological, biochemical, and biomolecular
analyses.

Materials and methods

Ethics statement
All procedures involving animals were approved and conducted in accordance with the guidelines set by the Animal Experiments Committee (DEC) of Utrecht University (Experimental numbers: 2012.III.07.065, 2013.III.02.017, and 2013.II.12.126), as required by Dutch regulation.

Isolation and culture of nucleus pulposus cells

Nucleus pulposus tissue was separated from early degenerated IVDs (Pfirrmann grade 2) of twelve laboratory beagle dogs; care was taken to avoid the transitional zone. Tissue was washed with hgDMEM + Glutamax + pyruvate (31966, Invitrogen, Paisley, UK) + 1% penicillin/streptomycin (p/s) (P11-010, PAA Laboratories GmbH, Piscataway, NJ, USA) and digested with 0.15% pronase (11459643001, Roche Diagnostics, Indianapolis, USA) for 45 minutes at 37°C and subsequently digested overnight with 0.15% collagenase II (4176, Worthington, Lakewood, NJ, USA) at 37°C. NPCs were filtered over a 70 μm filter, centrifuged (5 min at 500 g), and cryopreserved at passage 0 (hgDMEM + Glutamax + 10% dimethyl sulfoxide (DMSO) + 10% fetal bovine serum (FBS) (High performance 16000-044, Gibco, Bleiswijk, The Netherlands)) until further use. NPCs were expanded in expansion medium (hg DMEM + Glutamax + pyruvate (Invitrogen), 10% FBS, 1% p/s, 20 mM ascorbate-2-phosphate (A8960, Sigma-Aldrich, Saint Louis, MO, USA), 0.02 mM dexamethasone (D1756, Sigma-Aldrich), 1 ng/ml bFGF (PHP105, AbD Serotec, Oxford, UK), 0.5% Fungizone (15290-018, Invitrogen) in 175 cm² cell culture flasks (660175, Greiner bio-one, Cellstar, Alphen aan den Rijn, The Netherlands) until passage 2.

Cells (P2) were pooled to yield 5 different NPC donor groups and pelleted in ultra-low attachment 96 wells plates (Corning Costar 7007, Sigma-Aldrich) by centrifugation at 185 g for 8 minutes. Each pellet contained 200,000 NPCs and was cultured in 200 μl chondrogenic culture medium for 28 days (5% CO₂, 20% O₂). Standard chondrogenic medium (hg DMEM+Glutamax (Invitrogen), was supplemented 1% insulin-transferrin-selenium (ITS) + premix (354352 Corning, Tewksbury, MA, USA), 20 mg/ml proline (P5607 Sigma-Aldrich), 1% p/s, 0.5% Fungizone, 0.02 mM dexamethasone, 20 mM ascorbate-2-phosphate) and remained untreated (negative control) or was supplemented with 10 or 100 ng/ml rhBMP-7 (mammalian cell derived; 354-BP-010 R&D Systems Europe Ltd, Oxon, UK). Media were renewed twice weekly, collected per week, and stored at -80°C for analysis of GAG content.
Glycosaminoglycan and DNA content of NPC pellets and glycosaminoglycan content of culture media

At days 7 and 28, two NPC pellets per donor and condition were digested overnight at 60°C in papain (250 μg/ml papain (P3125-100 mg, Sigma-Aldrich) + 1.57 mg cysteine HCl (C7880, Sigma-Aldrich)). The 1,9-dimethylmethylene blue (DMMB) assay was used to quantify GAG content of the pellets and media. GAG concentrations were calculated by using chondroitin sulfate from shark cartilage (C4384,Sigma-Aldrich) as a standard and the absorbance was read at 540/595nm. The Quant-it™ dsDNA Broad-Range assay kit in combination with a Qubit™ fluorometer (Invitrogen) was used in accordance with the manufacturer’s instructions to determine the DNA content of the NPC pellets.

RNA isolation and quantitative RT-PCR of nucleus pulposus cell pellets

At days 7 and 28, RNA was isolated from two pellets per donor and condition and pooled. After crushing the pellets with a pellet pestle (9951-901, Argos Technologies, Elgin, IL, USA), total RNA was isolated by using the RNeasy microkit according to the manufacturer’s instructions. After on-column DNase-I digestion (Qiagen RNase-free DNase kit), RNA was quantified by using a NanoDrop 1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). The iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) was used to synthesize cDNA. Quantitative PCR (qPCR) was performed using an iCycler CFX384 Touch thermal cycler, and IQ SYBRGreen Super mix (Bio-Rad). qPCR was performed to assess the effects of rhBMP-7 at gene expression levels on: 1) extracellular matrix (ECM) anabolism; aggregcan (ACAN), collagen type 2 alpha 1 (COL2A1), collagen type 1 alpha 1 (COL1A1); 2) proliferation; cyclin-D1 (CCND1); 3) ECM catabolism; a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS5), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinase 1 (TIMP1); 4) apoptotic markers: B-cell lymphoma 2-associated X (BAX), B-cell lymphoma 2 (BCL2) and caspase 3 (CASP3); and 5) BMP signaling: BMP receptor 1A (BMPR1A), BMP receptor 1B (BMPR1B), BMP receptor 2 (BMPR2), inhibitor of DNA binding 1 (ID1), noggin (NOG) (Additional file 1). All dog-specific primers were designed in-house using Perlprimer except for MMP13. Primer specificity was evaluated with BLAST, and the designed amplicon was tested for secondary structures using MFold. Primers were purchased from Eurogentec, Maastricht, The Netherlands. Amplification efficiencies ranged from 86% to 119%. Relative expression levels were determined by the efficiency-corrected delta-delta Ct (ΔΔCt) method. Ct values of each target gene were normalized by the mean Ct value of 4 reference genes (ΔCt), i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S19 (RPS19), succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA), and hypoxanthine-guanine phosphoribosyltransferase (HPRT), whereas the mean Ct of all conditions for each target gene was used as a calibrator (ΔΔCt).
Histopathological evaluation of nucleus pulposus cell pellets
Safranin-O/fast green staining was performed to evaluate the presence of GAG deposition at a histopathological level. Two pellets per donor and condition were fixed overnight in neutral buffered formaldehyde 4% (Boom B.V., Meppel, The Netherlands) supplemented with 1% eosin (115935, Merck, Schiphol-Rijk, The Netherlands). Subsequently, the pellets were first embedded in alginate and thereafter in paraffin. Sections (5 μm) were stained with Mayer’s hematoxylin (3870, Avantor Performance Materials, Center Valley, PA), safranin-O (58884, Sigma-Aldrich) and, as a counterstaining, fast green (F7252, Sigma-Aldrich).

Experimental animals
Seven intact male beagle dogs with a median age of 1.3 years (range 1.1 – 1.8) and a median weight of 11.7 kg (range 10.2 – 12.8) were purchased from Harlan (Gannat, France). All dogs underwent a general, orthopedic, and neurologic examination by a board-certified veterinary surgeon (BM). The study was set up following a randomized block design. Single bolus injections of 2.5, 25, 250 μg rhBMP-7 (mammalian cell derived, CYT-276, ProSpec-Tany TechnoGene Ltd, Ness-Ziona, Israel), and a sucrose buffer (sham) were injected into the NPs in the T13-S1 spinal segments in a balanced Latin square design. IVDs adjacent to those injected with 250 μg of rhBMP-7 remained untreated. Preliminary studies in cadaveric spines showed that a volume of 40 μl could be injected into the NP without considerable resistance (unpublished data). The highest dosage of rhBMP-7 was constrained by the highest possible concentration that could be accomplished via dialysis, i.e. 250 μg in 40 μl.

Preparation of rhBMP-7 for in vivo application
Prior to the in vivo studies, the biological activity of rhBMP-7 from different manufacturers was determined in an alkaline phosphatase (ALP) activity assay in ATDC5 cells, amongst them rhBMP-7 from R&D and ProSpec-Tany. rhBMP-7 from ProSpec-Tany showed highest biological activity and was further chosen to be employed in the in vivo studies (Additional file 2). RhBMP-7 (CYT-276, ProSpec-Tany TechnoGene Ltd) was reconstituted in 60 μl sterile water. This solution was dialyzed against a buffer solution containing 17% sucrose, 20% mannitol, 332 mM glycine and 0.8% Tween 20, using a slide-A-Lyzer dialysis cassette (66454, Thermo Fisher Scientific Inc., Rockford, IL, USA) with a molecular weight cutoff of 10 kDa overnight with 4 buffer changes. The final solution containing 300 μg of rhBMP-7 (calculated amount) was freeze-dried overnight and reconstituted in sterile water, to achieve a final concentration of 250 μg rhBMP-7 in 40 μl buffer solution containing 1% sucrose, 1.2% mannitol, 20 mM glycine, and 0.05% Tween 20. Activity of the dialysate was shown to be retained in vitro through its capacity to induce ALP production in mice ATDC5 cells. Additional file 2 shows the biologic activity of the dialyzed rhBMP-7 compared with
Intradiscal application of rhBMP-7

Magnetic resonance (MR) imaging

MR images of the lumbar vertebral column were obtained in fully anesthetized dogs prior to surgery (t₀) and at 6 (t₁), 12 (t₁₂), and 24 (t₂₄) weeks after surgery. Dogs were pre-medicated with intravenous dexmedetomidine 10 μg/kg and butorphanol 0.1 mg/kg i.v., and anesthesia was induced with a continuous rate infusion of propofol (3-4 mg/kg) and dexmedetomidine 1 μg/kg c.r.i. A laryngeal mask was inserted, and anesthesia was maintained with isoflurane (2-3%) in a 1:1 oxygen/air mixture. Prior to the first MRI, a blood sample was drawn from the jugular vein to assess white blood cell count and differentiation, to exclude systemic inflammation. MRI was performed with a 1.5 Tesla scanner using a Spine array coil (Philips Healthcare, Best, The Netherlands). Sagittal T2-weighted (T2W) images were acquired using a turbo-spin echo (TSE) pulse sequence with the following parameters: repetition time (TR) = 2557 ms, echo time (TE) = 100 ms, field of view (FOV) = 200 mm, acquisition matrix = 332 x 306, slice thickness = 2 mm, number of slices = 13. For T2 mapping acquisition parameters were as follows: TR = range 2000 – 4000 ms, TE = 12.5 ms to 100 ms in 12.5 ms increments, FOV = 250 mm, acquisition matrix = 416 x 200, slice thickness = 2 mm, number of slices = 11. Sagittal T₁p weighted imaging was performed using a spin-lock-prepared sequence with a 3D multi-shot ultrafast gradient echo (T₁-TFE) readout with the following parameters: TR = 8.3 ms, TE = 4.3 ms, FOV = 250 mm, acquisition matrix = 416 x 378, slice thickness = 2 mm, number of slices = 25, TFE factor = 64, flip angle = 10°, shot interval = 2000 ms. To allow quantitative T₁p mapping, data were acquired five times, each with a different spin-lock time (TSL); 1, 10, 20, 40 and 80 ms. Spin-lock amplitude was set to 500 Hz.

T₂ and T₁p quantification

Mid-sagittal slices of T2W images were used to evaluate the grade of degeneration at all time points. Lumbar IVDs were assessed by a veterinary radiologist that was blinded to treatment allocation, according to the Pfirrmann classification validated for dogs by Bergknut et al. Only lumbar IVDs with a Pfirrmann score II at t₀ were included for injection. Disc height index (DHI) was calculated at all time points on T2W images for each IVD according to the method described by Masuda et al. In short, DHI was calculated by averaging widths of the dorsal, middle, and ventral parts of the vertebral disc and dividing that by the average of dorsal, middle, and ventral body heights of the adjacent cranial and caudal vertebrae. To calculate the DHI of L7 – S1, the average body height of only the cranial vertebra (L7) was used, as S1 has a different shape than the lumbar vertebrae. For the analysis of T₂ and T₁p values, an oval shaped region of interest (ROI) was manually segmented on mid-sagittal sections, to select NP tissue in each IVD in the free open-source DICOM viewer Osirix (Pixmeo, Geneva, Switzerland). ROIs were exported to and
analyzed with Wolfram Mathematica 10.0 (Wolfram Research, Champaign, IL, USA). T2 and T1\(\rho\) values were computed by calculating the mean signal intensity (S) in each ROI, and by fitting these intensity data into equations:

\[
S(TE) = S_0 e^{-TE/T2}
\]

or

\[
S(TSL) = S_0 e^{-TSL/T1\rho}
\]

respectively, using the Levenberg-Marquardt nonlinear least-squares method implemented in Mathematica. \(S_0\) denotes the equilibrium magnetization, whereas \(S(TE)\) and \(S(TSL)\) indicate the T2- and T1\(\rho\)-prepared signals, respectively.

**Surgical procedure**

The anesthesia protocol for MR imaging and surgery was identical. Analgesia was provided pre-operatively by intravenous methadone 0.5 mg/kg and carprofen 4 mg/kg, and peri-operatively by a continuous rate infusion of a combination of fentanyl (loading dose 10 \(\mu\)g/kg, 15-20 \(\mu\)g/kg/hr) and ketamine (0.5 mg/kg loading dose, 10 \(\mu\)g/kg/min). Dogs were positioned in right recumbence to expose and inject T13-L6 via a left lateral approach, and subsequently in ventral recumbence to expose and inject L6-S1 via a dorsal approach. A 100 \(\mu\)l gastight syringe (7656-01 Model 1710 RN) with a 27G needle (25 mm, 12° beveled point; Hamilton Company USA, Reno, Nevada, USA) was used to inject 40 \(\mu\)l of the BMP-7 containing solutions or control into the NP under magnified vision (3.3x). Location of the tip of the needle in the NP was estimated by the distance of passage through the annulus fibrosus (AF) (1 cm), while encountering steady resistance. When the NP was reached, the resistance decreased and the volume of 40 \(\mu\)l could be easily injected. The needle was retracted slowly to allow the AF puncture site to close, and the site was inspected for extrusion of the administered compound and rinsed with 0.9% NaCl. Postoperative pain management in all dogs consisted of intramuscular methadone 0.3 mg/kg six times a day and subcutaneous carprofen 4 mg/kg once a day during the first 24 hours, and tramadol 2-5 mg/kg administered orally four times a day, and carprofen 4 mg/kg orally the following 7 and 10 days, respectively. All dogs were treated postoperatively with antibiotics (amoxicillin/clavulanic acid 12.5 mg/kg twice a day) during 5 days. Dogs were monitored daily throughout the study to assess pain symptoms according to the short form of the Glasgow composite pain scale.\(^{29}\) Dogs that showed signs of pain, received carprofen and/or tramadol.

**Radiographic imaging and computed tomography (CT)**

Radiographs and CT-scans of the T11 – S3 segment were obtained post mortem (t\(24\)) and were evaluated by an independent veterinary radiologist for new bone formation, end plate sclerosis and disc protrusion (only CT). Lateral and dorsoventral radiographs were obtained with a digital radiography system (Philips digital Rad TH, Eindhoven, The
Netherlands) using 50 kVp and 5 mA. Transverse CT images were acquired with a third-generation single-slice helical CT-scanner (Philips Secura, Eindhoven, The Netherlands). Contiguous 2 mm thick slices with 1 mm overlap were obtained from T11 – S3 with exposure settings of 120 kV and 260 mA.

Sample collection, macroscopic grading, and histopathological grading
Dogs were euthanized 24 weeks post-injection by way of sedation with intravenous dexmedetomidine 0.04 mg/kg, followed by pentobarbital 200 mg/kg. Subsequently, the vertebral column (T12 – S1) was harvested by using an electric multipurpose saw (Bosch, Stuttgart, Germany). All muscles were removed and the lumbar vertebrae were transected transversely with a band saw (EXAKT tape saw, EXAKT Advanced Technologies GmbH, Norderstedt, Germany) resulting in nine spinal units (½ vertebra – endplate – IVD – endplate – ½ vertebra). A diamond band pathology saw (EXAKT 312 saw; EXAKT diamond cutting band 0.1 mm D64; EXAKT Advanced Technologies GmbH, Norderstedt, Germany), was used to transect these units sagitally into two identical parts. From one part, the endplate and vertebra were removed with a surgical knife and the remaining IVD tissue, containing NP and AF, was snap frozen in liquid nitrogen and stored at -80°C for biochemical and biomolecular analyses. The other part was photographed (Olympus VR-340, Hamburg, Germany) for macroscopic evaluation (Thompson score) and fixed in 50 ml of 4% buffered formaldehyde (Klinipath, Duiven, The Netherlands) at 4°C for 14 days. Images of the IVD segments were evaluated blinded and in random order by two independent blinded investigators according to the Thompson grading scheme, which has been validated in dogs.30

Samples were decalcified in 35% formic acid and 6.8% sodium formate in a microwave oven (Milestone Microwave Laboratory Systems, Italy) overnight at 37°C, during 7 nights and embedded in paraffin.31 Sections (5 μm) were stained with hematoxylin (109249, Merck) and eosin (115935, Merck) and with picrosirius red (saturated aqueous picric acid: 36011, Sigma-Aldrich, sirius red: 80115, Klinipath)/alcian blue (alcian blue: 05500, Sigma-Aldrich; glacial acetic acid: 100063, Merck) and evaluated according to a grading scheme according to Bergknut et al.21 Histological sections were scored blinded and in random order by two independent investigators (NW, SP) using an Olympus BX41 microscope. In case of disagreement, samples were also scored by a board-certified veterinary pathologist (GG).

RNA isolation and qPCR of NP and AF
Cryosections (60 μm) of the IVD of the remaining spinal unit (endplate – IVD) were cut with a cryostat (Leica CM1800 cryostat, Leica Microsystems Inc., Bannockburn, USA) and collected on RNA-se free glass slides. The NP and AF tissues were separated and half of the
tissues were collected in 400 μl and 750 μl cOmplete lysis M EDTA-free buffer (Roche Diagnostics Nederland B.V., Almere, The Netherlands) respectively, and stored at -80°C until biochemical analysis. The other halves were collected in 300 μl RLT buffer containing 1% β-mercapto-ethanol (Qiagen, Venlo, The Netherlands) and stored at -80°C until biomolecular analysis. Total RNA was isolated by using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, The Netherlands). The incubation period with proteinase K was reduced to five minutes to increase RNA yield. RNA quantification and cDNA synthesis were performed in a similar way as described for the NPC pellets in vitro. Reference genes and the subset of target genes were similar to those determined in vitro.

Glycosaminoglycan, DNA and collagen assays of nucleus pulposus and annulus fibrosus
To measure GAG and DNA content, the NP and AF samples were homogenized in cOmplete lysis M EDTA-free buffer in a TissueLyser II (Qiagen) for 2x 30 s at 20 Hz. The supernatant and pellet of each NP and AF were digested overnight in papain and measurements were performed as described in vitro. Collagen was quantified in the pellets of the NP and AF by using a hydroxyproline assay.32 Samples were freeze-dried overnight, hydrolyzed at 108 °C overnight in 4M NaOH, centrifuged (15 s at 14000 g) and stored at -20 °C. Prior to measurements, samples were centrifuged (15 s at 14000 g) once more, chloramine T reagent (2426, Merck) was added and samples were allowed to shake for 20 min at 170 rpm. Freshly prepared dimethylaminobenzaldehyde (3058 Merck) was added, and samples were incubated for 20 minutes at 60 °C. The absorbance was read at 570nm and collagen content was calculated from the hydroxyproline content by multiplying with a factor 7.5.32 DNA and collagen content in the supernatants were negligible and therefore not included in the calculations. Total GAG and collagen content were normalized to DNA content in the sample.

Statistical analysis
All data were analyzed by using R statistical software, package 3.0.2.33 Residual plots and quantile-quantile (Q-Q)-plots were used to check normality of the data. In case of violation, data were logarithmically transformed. If non-normality remained after transformation, nonparametric tests were employed. In vitro, cumulative GAG release, GAG and DNA content, the GAG/DNA ratio and ΔCt values for the investigated target genes, were statistically evaluated by using the nonparametric Kruskal-Wallis test, followed by a Mann-Whitney U-test. The effect of the injected treatments in vivo on GAG, DNA and collagen content, DHI, and T1ρ and T2 values was analyzed with a linear mixed effect model. A random effect ‘dog’ (dog 1-7), was incorporated to capture the correlation between multiple measurements within one dog. For GAG, DNA and collagen analysis, factors incorporated into the model as a fixed effect were ‘treatment’ (2.5 μg rhBMP-7, 25 μg rhBMP-7, 250 μg rhBMP-7, and sham), ‘tissue’ (NP and AF), and their interaction. For
DHI analysis ‘treatment’, ‘time’ (t₀, t₆, t₁₂, and t₂₄), and their interaction served as fixed effect factors. The Cox proportional hazards regression model was used to estimate the effect of the injected treatments on gene expression levels in vivo. Calculations were performed on the ratio of the Ct values for each target gene to the mean Ct value of the reference genes. Ct values ≥ 40 were right censored. Regression coefficients were estimated by the maximum likelihood method. Model selection was based on the lowest Akaike Information Criterion (AIC). Differences between treatments were considered significant if the confidence interval did not include 0, whereas hazard ratios were considered significant if the confidence interval did not include 1. For in vitro and in vivo analyses, confidence intervals were calculated and stated at the 99% confidence level to correct for multiple comparisons. Significant differences and the corresponding confidence intervals are represented in Additional file 3.

**Figure 1.** Glycosaminoglycan (GAG) release, GAG, DNA and GAG/DNA content in cultured nucleus pulposus cells (NPCs) treated with 10 or 100 ng/ml rhBMP-7. a. NPC pellets treated with rhBMP-7 show a significant dose-dependent increase in cumulative GAG release into the medium compared with the negative control. b. Regardless of the culture condition, DNA content of the NPC pellets was significantly lower compared with DNA DNA_{-7} showed a significantly higher DNA content at day 28 compared with the negative control and the 10 ng/ml rhBMP-7 treated NPC pellets. c, d. A significant increase in GAG content and GAG/DNA at day 28 was shown in the rhBMP-7 100 ng/ml treated NPC pellets compared with the negative control and the 10 ng/ml rhBMP-7 treated NPC pellets. Data are expressed as mean ± standard deviation. **Indicates significant difference at a 99% confidence interval (CI); *indicates significant difference at a 98% CI; # indicates significant difference at a 99% CI.
Results

Effect of rhBMP-7 on early degenerated canine NPC pellets in vitro

Cell maintenance and increased GAG release and content by rhBMP-7

NPC pellets treated with 10 and 100 ng/ml rhBMP-7 showed a dose-dependent significant increase in cumulative GAG release into the medium compared with the negative control (Figure 1a). Regardless of the culture condition, DNA content of the NPC pellets was significantly lower compared with DNA content at day 0. Treatment with 100 ng/ml rhBMP-7 resulted in a significantly higher DNA content at day 28 compared with the negative control and the 10 ng/ml rhBMP-7 treated NPC pellets (Figure 1b). A significant increase in GAG content and GAG/DNA at day 28 was shown in the rhBMP-7 100 ng/ml treated NPC pellets compared with the negative control and the 10 ng/ml rhBMP-7 treated NPC pellets. Safranin-O/Fast green staining of the NPC pellets at day 28 showed a higher GAG deposition in the matrix of the pellets treated with 100 ng/ml rhBMP-7 compared with the negative control and the 10 ng/ml rhBMP-7 treated pellets (Figure 2).

Pro-anabolic and anti-apoptotic effect of rhBMP-7

Expression of ECM genes ACAN and COL2A1 was significantly upregulated at day 7 in the 100 ng/ml rhBMP-7-treated NPC pellets compared with the negative control and the 10 ng/ml rhBMP-7 treated NPC pellets (Figure 3a). COL2A1 expression was significantly upregulated in the 100 ng/ml rhBMP-7 treated NPC pellets compared with the negative control at day 28. Expression of COL1A1 did not differ between conditions at any of the time points. Relative expression of the proliferative marker CCND1 was significantly upregulated in all conditions at day 28 compared with day 7, while it was also significantly higher in the 100 ng/ml rhBMP-7 treated NPC pellets at day 7 compared with the negative control. Furthermore, CCND1 expression levels were significantly higher in the NPC pellets treated with 10 ng/ml rhBMP-7 at day 28 compared with the negative control (Figure 3a). Gene expression of the catabolic gene ADAMTS5 was significantly lower in the 100 ng/ml rhBMP-7 treated NPC pellets compared with the 10 ng/ml rhBMP-7 treated NPC pellets at day 28 (Figure 3b). In the NPC pellets treated with 10 and 100 ng/ml rhBMP-7, gene expression of the catabolic gene MMP13 was significantly lower at day 28 compared with day 7 (Figure 3b). The relative gene expression of the anti-catabolic gene TIMP1 was significantly higher in the negative control and 10 ng/ml rhBMP-7 treated NPC pellets at day 7 compared with day 28 (Figure 3b). The BAX/BCL2 ratio in the 100 ng/ml rhBMP-7 treated NPC pellets was significantly higher at day 28 compared with day 7 (Figure 3c). Relative expression of the apoptotic marker CASP3 was significantly downregulated at day 28 in the 10 and 100 ng/ml rhBMP-7 treated NPC pellets (Figure 3c). Altogether, these results show a stimulatory effect of 100 ng/ml rhBMP-7 on matrix anabolism, that seemed most profound at day 7, and an anti-apoptotic effect, most profound at day 28.
Intradiscal application of rhBMP-7

Figure 2. Representative histological images of nucleus pulposus cells (NPCs) cultured in pellets for 28 days stained with Safranin-O/Fast green. NPC pellets treated with 100 ng/ml rhBMP-7 showed a positive Safranin-O/Fast green staining for GAGs and an increased size compared with the negative control and the 10 ng/ml rhBMP-7-treated pellets.
Figure 3. Relative gene expression of relevant target genes in nucleus pulposus cells (NPCs) cultured in pellets at day 7 and 28. A. Anabolic markers: aggrecan (ACAN), collagen type 2 alpha 1 (COL2A1), cyclin-D1 (CCND1); B. Catabolic markers: a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMT55), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinase 1 (TIMP1); C. Apoptotic markers: caspase 3 (CASP3), B-cell lymphoma 2-associated X (BAX), and B-cell lymphoma 2 (BCL2) in non-treated (negative control) and 10 and 100 ng/ml rhBMP-7 treated NPC pellets. Data are expressed as relative expression ± standard deviation, except for the BAX/BCL2 ratio. ** Indicates significant difference at a 99% confidence interval (CI); * indicates significant difference at a 98% CI; δ indicates a significant difference at a 97% CI; ● indicates significant difference at a 96% CI.
Intradiscal application of rhBMP-7 in laboratory beagle dogs

No regenerative changes of rhBMP-7 at IVD level on macroscopy and T2W MR images

All dogs recovered uneventfully from surgery and were ambulant the next day. During follow-up all dogs were treated with analgesics and antibiotics in accordance with the protocol described under ‘surgical procedure’, and none of the dogs required additional medication. Before surgery a total of 63 IVDs were graded on T2W MR images (t0). Sixty-two out of 63 IVDs were assigned a grade II according to the Pfirrmann system, whereas 1 IVD was assigned a grade III. A total of 42 grade II IVDs were injected. In 61/63 IVDs, the Pfirrmann scores remained unchanged over time. The IVD that was assigned a grade III at t0, was assigned a grade II at all subsequent time points, and 1 IVD, treated with the sucrose buffer, that was scored a grade II at t0, was re-graded a Pfirrmann score III. Post-mortem, 62/63 IVDs were assigned a Thompson score grade II, consistent with early IVD degeneration, whereas 1 IVD, treated with 250 μg rhBMP-7, was assigned a grade IV.

Extradiscal new bone formation after intradiscal injection of 25 and 250 μg rhBMP-7

In 1/63 IVDs, extensive new bone formation was noted on the ventral aspect on MRI at 6, 12 and 24 weeks post-surgery (t6, t12, and t24). Ventral bone formation was noted on macroscopy post-mortem in this particular IVD and in one other. The IVD that was scored a Thompson grade IV showed rupturing of the dorsal AF, with NP material extending into the spinal canal, irregularity of the endplates, and focal sclerosis in the subchondral bone (Figure 4d).

CT images of this dog showed symmetric extensive bulging of the L7-S1 IVD (lumbosacral junction) that had been injected. In 4 out of 63 IVDs, radiographs and CT images revealed margined extradiscal new bone formation ventrally, laterally (left) and ventro-laterally (right side) in combination with mild sclerosis of the underlying bone post-mortem (Figure 4). In 3 out of 42 IVDs these findings were associated with treatment with 250 μg rhBMP-7, in 1 out of 42 with 25 μg rhBMP-7. In 2 out of 63 IVDs mineralization was shown on CT images; one of these IVDs had been injected with 2.5 μg rhBMP-7, whereas the other one had not been injected. DHI, T2-values, as well as T1p-values did not show significant differences between treatments over time.

Anti-apoptotic effect of 250 μg rhBMP-7

All IVDs were evaluated histopathologically according to the grading scheme according to Bergknut et al.21 The median histological grade was 13 (range: 8 to 20) and there were no significant differences between the treatments. In the IVD, treated with 250 μg rhBMP-7, that was scored a Thompson grade IV, NP material and fibroblasts were present in the outer AF. Furthermore, in 2 IVDs treated with 250 μg rhBMP-7, bone formation was confirmed.
Figure 4. Extradiscal bone formation after intradiscal injection of recombinant human bone morphogenetic protein-7 (rhBMP-7). Radiography, computed tomography (CT), macroscopy, and histology of intervertebral discs (IVDs) injected with 2.5 µg (a), 25 µg (b), and 250 µg (c, d) rhBMP-7. A. Unremarkable findings after injecting 2.5 µg rhBMP-7 into the intervertebral disc (IVD). The histological image shows a typical example of an early degenerated nucleus pulposus (NP), consisting of clusters of chondrocyte-like cells. B. On the left lateral site of the vertebral column extradiscal new bone formation is shown on the radiograph and CT image (white arrow) next to the IVD injected with 25 µg rhBMP-7. Histology was consistent with early degenerative changes in the IVD as shown in A. C. Extradiscal new bone formation ventrally of the vertebral column and sclerosis of the ventral vertebral cortex is shown on radiography (white arrow), CT, macroscopy and histology (double asterisk) after injecting 250 µg rhBMP-7 into the IVD. D. Rupturing of the dorsal annulus fibrosus (AF), and protrusion of NP material into the spinal canal (black arrow) on CT, macroscopy and histology image of an IVD injected with 250 µg rhBMP-7. Irregularity of the endplates, and focal sclerosis in the subchondral bone (white arrowheads) was seen on macroscopy and confirmed on histology (not shown in this image). L = lumbar vertebra, F = facet joint, SC = spinal cord, Tr = transverse process, I = ilium, NP = nucleus pulposus, EP = endplate, AF = annulus fibrosus, S = sacral vertebra, SC = spinal cord, Tr = Transverse process.
The $BAX/BCL2$ ratio in the IVDs (NP and AF) treated with 250 $\mu$g rhBMP-7 were significantly upregulated compared with the IVDs (NP and AF) treated with the sham and 25 $\mu$g rhBMP-7, suggestive of an anti-apoptotic effect of 250 $\mu$g rhBMP-7. Relative gene expression of $CASP3$ in the NP was significantly higher than in the AF in all treatments, indicative of a higher apoptotic rate in the NP (Figure 5).

**No anabolic effects of rhBMP-7 on extracellular matrix**

No significant differences in GAG corrected for DNA, nor in collagen corrected for DNA were found between the treatments in the NP or the AF (Figure 6a). Regardless of the treatment, GAG/DNA in the NP was significantly higher than in the AF, consistent with the known physiological differences in matrix composition at protein level between the NP and the AF. These physiological differences were also reflected by relative gene expression, as gene expression levels of $COL2A1$ were significantly higher in the NP than in the AF, and the expression levels of $COL1A1$ were significantly lower in the NP than in the AF (Figure 6b). Relative gene expression of anabolic ($ACAN$, $COL2A1$, $COL1A1$), catabolic ($MMP13$) and anti-catabolic ($TIMP1$) genes did not significantly differ between treatments. Gene expression of $ADAMTS5$, and BMP receptors $BMPR1A$, and $BMPR1B$ were below the detection level in both the NP and the AF, independent of treatment. BMP-7 receptor $BMPR2$, and the downstream target of the BMP-7 signaling pathway gene $ID1$, did not significantly differ between the treatments. Relative gene expression of BMP signaling inhibitor $NOG$ varied a lot regardless of the groups and was significantly higher in IVDs treated with 25 $\mu$g compared with 2.5 $\mu$g and sham treated IVDs; relative gene expression of $NOG$ is reported in Additional file 4.

**Figure 5.** Relative gene expression of genes associated with apoptosis. Relative gene expression of caspase 3 ($CASP3$), B-cell lymphoma 2-associated X ($BAX$)/B-cell lymphoma 2 ($BAX/BCL2$) ratio in the nucleus pulposus (NP) (open bars) and the annulus fibrosus (AF) (filled bars) injected with a sucrose buffer (sham), 2.5, 25, or 250 $\mu$g rhBMP-7, were indicative of an anti-apoptotic effect of 250 $\mu$g rhBMP-7. The sham treated NP was used as a reference, and was set at 1 (dashed line). Data are expressed as n-fold change ± standard deviation. *Indicates a significant difference at a 96% CI; **indicates a significant difference at a 99% confidence interval (CI);
Figure 6. Relative gene expression of matrix-related target genes and DNA, glycosaminoglycan (GAG), and collagen content in intervertebral discs (IVDs) injected with rhBMP-7. 

a. GAG/DNA and collagen/DNA did not significantly differ between the treatments. GAG/DNA in the nucleus pulposus (NP) (open bars) was significantly higher than in the annulus fibrosus (AF) (filled bars).

b. Relative gene expression of collagen type 2 alpha 1 (COL2A1) and collagen type 1 alpha 1 (COL1A1) did not significantly differ between treatments. Gene expression levels of COL2A1 were significantly higher in the nucleus pulposus (NP) than in the annulus fibrosus (AF), whereas the expression levels of COL1A1 were significantly lower in the NP than in the AF. GAG/DNA and collagen/DNA are expressed as mean ± standard deviation, and COL2A1 and COL1A1 as relative expression ± standard deviation. ** Indicates a significant difference at a 99% confidence interval (CI)
Discussion

In this study we first showed that 100 ng/ml rhBMP-7 was biologically active in mildly, spontaneously degenerated canine NPCs in vitro. Treatment with 100 ng/ml rhBMP-7 stimulated matrix production by canine NPC pellets isolated from degenerated discs, reflected by a significant higher expression of ACAN and COL2A1 seen at day 7 and a significant increase in GAG release and deposition mainly seen at day 28 of culture. Treatment with 10 ng/ml rhBMP-7 showed a significant increase in GAG release compared with the negative control, but did not result in increased GAG deposition. This is most likely due to a suboptimal balance in GAG and collagen synthesis and breakdown, resulting in the inability to deposit GAGs in a newly formed collagen network. During pellet culture, in the absence of rhBMP-7, DNA content decreased significantly over time. NPC pellets treated with rhBMP-7 showed preservation of the DNA content at initial levels, with a significant upregulation of the biomolecular proliferative marker CCND-1 limited to day 7, and a significant downregulation of the pro-apoptotic marker CASP3 and an increase of the BAX/BCL2 ratio at day 28. Our findings are consistent with the pro-anabolic and anti-apoptotic properties of rhBMP-7 that have been shown in vitro in NP cells of different species, i.e. bovine, rabbit, human.

Next, we aimed at determining the safe and optimal regenerative dose of rhBMP-7 for intradiscal application in a spontaneous canine disc degeneration model. However, intradiscal injection of a wide dose range of rhBMP-7 (2.5 – 250 μg) did not result in regeneration of the canine IVD. In contrast to what has been described in rabbit models, extracellular matrix production in the NP and the AF did not differ between treatments. It should be noted that our data analysis is limited by the absence of wet weight data of the samples and that the necessary correction for DNA content might have leveled out the effects between treatments. However, disc height index, T2, and T1p maps and histological grading did not differ between treatments confirming the biochemical data and hence corroborating the absence of a regenerative effect after intradiscal application of a wide range of dosages of rhBMP7. Contrasting findings between the current canine model based on spontaneous degeneration and the healthy or rabbit models of induced IVD degeneration, are most likely related to differences in disc size and cell types that populate the NP. Cell type variation related to differences in genetic background may play a role, with a concomitant difference in degenerative, as well as regenerative pathways. Notochordal cells (NCs) are thought to play a key role in regeneration. In rabbits, NCs are retained in the NP at least until 12 months of age, while in humans NCs are lost before 4-10 years of age, and in chondrodystrophic dogs, e.g. beagles, before 1 year of age. Although induction of rabbit IVD degeneration by trauma is accompanied by an inflammatory response, and ultimately results in decreased amounts of extracellular matrix, a clear loss in disc height, and replacement of notochordal cells by chondrocyte-
like and fibroblast-like cells, persistence of notochordal cells cannot be ruled out.

Nevertheless, early degenerated canine NP cells in culture respond in a similar way to rhBMP-7 as rabbit NP cells, also suggestive of differences in rhBMP-7 activity in vitro and in vivo in the canine model. The anabolic and anti-apoptotic effect of rhBMP-7 on the tissue cells is mediated via specific BMP receptors that activate the intracellular signaling protein SMAD1/5/8. The latter then forms a complex with SMAD4, and the complex translocates into the nucleus and regulates transcription of target genes. BMP-7 in addition, upregulates natural BMP antagonists, e.g. NOG, that block the binding sites of the BMP receptors, thereby bringing rhBMP-7 in an inactive state. Relative gene expression of NOG was significantly upregulated in IVDs treated with 25 μg vs 2.5 μg and sham treated IVDs, indicating that BMP antagonists may indeed play a role in the regulation of in vivo BMP7 signaling. Given that the BMP signaling pathway is complicated, we can only speculate on the possible confounding effects limiting the biological effect of rhBMP-7 in vivo after 6 months follow-up, including availability of BMP receptors, activity of NOG, and the presence and ability of proteases in the degenerated IVD to degrade rhBMP-7. Another explanation for the differences in vitro and in vivo, was the addition of rhBMP-7 biweekly in vitro, while a single dose was applied intradiscally in vivo. Protein activity in vivo is likely to be lost quickly, due to a short biological half-life and by diffusion out of the tissue. The bioavailability of rhBMP-7 in vivo could be increased by using controlled-release systems, e.g. hydrogels or microspheres, that allow a sustained release of rhBMP-7 over a prolonged period of time, or by vector-mediated introduction of BMP-7 encoding genes.

Strikingly, mild to severe extradiscal new bone formation was seen after intradiscal administration of 25 μg and 250 μg rhBMP-7 in our canine model. Induction of bone formation requires three essential components: an osteo-inductive signal, a substratum and interactive host cells. The surgical procedure, associated with tissue injury, might have provided the chemotactic stimulus for the recruitment of required mesenchymal precursor cells. The application of rhBMP-7 might have provided the osteo-inductive stimulus for chondro-osteogenic differentiation, resulting in ectopic bone formation. Although suboptimal delivery of BMP-7 by our injection technique might have played a role, the rhBMP-7 might also have diffused out of the NP. This phenomenon was also described previously in a rabbit model in which osteophyte formation was induced by intradiscal injection of labelled mesenchymal stem cells. Diffusion of rhBMP-7 out of the IVD may have been enhanced by biomechanical forces and/or disorganization of the lamellar structure of the AF that are part of the early IVD degeneration process. Various dosages of rhBMP-7, ranging from 0.005 μg – 2 mg, with or without a carrier, have been reported to induce endochondral bone formation in extraskeletal sites (muscle, subcutis,
tendon, thyroid cartilage, and subdural space) and in several species, e.g. baboons, rats, and dogs. In all these studies the microenvironment appeared to be an essential component in tissue regeneration. Indeed, in humans rhBMP-7 is approved for the treatment of non-unions of long bones and the pelvis, and posterior lumbar fusion. rhBMP-7 is frequently used in man, however, adverse effects, such as ectopic bone formation, are only occasionally reported. Based on statements of researchers in reviews from 2008 and 2011, a multicenter clinical trial in the U.S. was started, in which BMP-7 was intradiscally injected into human patients with degenerative disc disease. However, thus far, results have not been published.

Conclusion
An anabolic effect of rhBMP-7 on extracellular matrix production of canine NPCs isolated from early degenerated IVDs was shown in vitro. Despite intradiscal administration of a wide dose range of rhBMP-7 (2.5 – 250 μg) in spontaneously early degenerated canine IVDs, we did not observe regenerative effects at the IVD level. In fact, injection of 250 μg rhBMP-7, and to a lesser extent 25 μg rhBMP-7, resulted in extensive extradiscal bone formation. Altogether, this study indicates that additional issues need to be addressed before intradiscally applied rhBMP-7 can be translated into a clinical application to treat low back pain in human and canine patients.

Acknowledgements
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References


Intradiscal application of rhBMP-7


## Additional file 1. Primers used for quantitative polymerase chain reaction

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Primers used for quantitative polymerase chain reaction analysis of reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S19 (RPS19), succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA), and hypoxanthine-guanine phosphoribosyltransferase (HPRT), and target genes aggrecan (ACAN), collagen type II (COL2A1), collagen type I (COL1A1), a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS5), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinase 1 (TIMP1), cyclin-D1 (CCND1), bone morphogenetic protein receptor type IA (BMPR1A), bone morphogenetic protein receptor type IB (BMPR1B), bone morphogenetic protein receptor type II (BMPR2), DNA-binding protein inhibitor 1 (ID1), noggin (NOG), B-cell lymphoma 2-associated X (BAX), B-cell lymphoma 2 (BCL2) and caspase 3 (CASP3).

Ann. = annealing; amp. = amplicon
**Additional file 2. Alkaline phosphatase activity rhBMP-7**

**Induction of alkaline phosphatase activity by rhBMP-7 from two different manufacturers in vitro**

rhBMP-7 of manufacturers R&D and ProsPec-Tany were evaluated for their activity to induce alkaline phosphatase (ALP) activity in mice ATDC5 cells. ATDC5 cells were cultured as described by Caron et al. and previously reported by us.\(^{50, 51}\) Cells were plated on 24-well plates (Greiner Cellstar, Alphen a/d Rijn, The Netherlands) at a density of \(4 \times 10^4\) cells/plate. After 24 hours, standard differentiation culture medium with 100 ng/ml BMP-7 was added, and cells were cultured for another 72 hours and total ALP activity was measured kinetically in the cell lysate in a kinetic microplate reader at 405 nm (Bio-Rad Benchmark, Veenendaal, The Netherlands). A volume of 50 μl of the cell lysate was incubated with 50 μl p-nitrophenyl phosphate for 20 minutes. rhBMP-7 of ProsPec-Tany induces a higher ALP activity compared with rhBMP-7 of R&D at the same concentration.

**Induction of ALP activity by rhBMP-7 dialysate in vitro**

After dialysis of the rhBMP-7 (ProsPec-Tany) biologic activity of the dialysate was determined by induction of ALP activity in mice ATDC5 cells (ProsPec-Tany) and compared to pre-dialyzed BMP-7 at the same concentration. As depicted in the adjacent figure the dialysate of ProsPec-Tany retained its biological activity.
### Additional file 3. Significant differences and the corresponding confidence intervals

**Table 1A.** Significant differences and confidence intervals of statistical analyses performed on data of the *in vitro* experiment. Figures correspond to figures shown in the main article.

#### Figure 1. DNA, GAG and GAG/DNA content in pellets containing nucleus pulposus cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vs</th>
<th>Difference in location*</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- control at day 7</td>
<td>day 0</td>
<td>1.09</td>
<td>0.36 – 1.73</td>
<td>99</td>
<td>0.008</td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 7</td>
<td>day 0</td>
<td>1.07</td>
<td>0.23 – 1.76</td>
<td>99</td>
<td>0.008</td>
</tr>
<tr>
<td>100 ng/ml rhBMP-7 at day 7</td>
<td>day 0</td>
<td>0.65</td>
<td>0.16 – 1.45</td>
<td>96</td>
<td>0.03</td>
</tr>
<tr>
<td>- control at day 28</td>
<td>day 0</td>
<td>1.41</td>
<td>0.91 – 2.00</td>
<td>99</td>
<td>0.009</td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>day 0</td>
<td>1.33</td>
<td>0.85 – 1.88</td>
<td>99</td>
<td>0.009</td>
</tr>
<tr>
<td>100 ng/ml rhBMP-7 at day 7</td>
<td>day 0</td>
<td>0.93</td>
<td>0.21 – 1.52</td>
<td>99</td>
<td>0.008</td>
</tr>
<tr>
<td>- control at day 7</td>
<td>- control at day 28</td>
<td>0.27</td>
<td>0.09 – 0.73</td>
<td>99</td>
<td>0.009</td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 7</td>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>0.54</td>
<td>0.04 – 0.74</td>
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<td>0.01</td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
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<td>-0.76 – -0.24</td>
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<td>0.009</td>
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<tr>
<td>- control at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
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<td>-0.88 – -0.30</td>
<td>99</td>
<td>0.009</td>
</tr>
<tr>
<td>Cumulative GAG release</td>
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<td></td>
<td></td>
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<tr>
<td>- control</td>
<td>10 ng/ml rhBMP-7</td>
<td>-25.40</td>
<td>-44.51 – -3.93</td>
<td>99</td>
<td>0.008</td>
</tr>
<tr>
<td>- control</td>
<td>100 ng/ml rhBMP-7</td>
<td>-125.26</td>
<td>-165.73 – -88.63</td>
<td>99</td>
<td>0.008</td>
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<td>10 ng/ml rhBMP-7</td>
<td>100 ng/ml rhBMP-7</td>
<td>-101.49</td>
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<td>0.008</td>
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<td>GAG content</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
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<td>-13.74 – -1.27</td>
<td>99</td>
<td>0.008</td>
</tr>
<tr>
<td>- control at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
<td>-8.2</td>
<td>-15.49 – -0.39</td>
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<td>0.008</td>
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<tr>
<td>- control at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
<td>-8.2</td>
<td>-15.49 – -2.48</td>
<td>99</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*GAG = glycosaminoglycan, DNA = deoxyribonucleic acid  
*Difference in location estimates the median of the difference between condition $x$ and condition $y$.  

---

**Intradiscal application of rhBMP-7**
Table 1B. Significant differences and confidence intervals of statistical analyses performed on data of the *in vitro* experiment. Figures correspond to figures shown in the main article.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
<th>vs</th>
<th>Condition</th>
<th>Difference in location*</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>ACAN</td>
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<td>2.67</td>
<td>0.74 – 3.90</td>
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<td>control at day 7</td>
<td>10 ng/ml rhBMP-7 at day 7</td>
<td>2.17</td>
<td>0.45 – 4.01</td>
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<td>0.008</td>
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<tr>
<td>COL2A1</td>
<td>control at day 7</td>
<td>100 ng/ml rhBMP-7 at day 7</td>
<td>4.15</td>
<td>0.94 – 5.51</td>
<td>99</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control at day 7</td>
<td>10 ng/ml rhBMP-7 at day 7</td>
<td>3.70</td>
<td>1.42 – 5.54</td>
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<td>0.009</td>
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<tr>
<td></td>
<td>control at day 28</td>
<td>100 ng/ml rhBMP-7 at day 28</td>
<td>3.92</td>
<td>0.84 – 7.92</td>
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<td>0.03</td>
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<td>CCND1</td>
<td>control at day 7</td>
<td>100 ng/ml rhBMP-7 at day 7</td>
<td>0.70</td>
<td>0.2 – 1.76</td>
<td>99</td>
<td>0.008</td>
<td></td>
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<td>10 ng/ml rhBMP-7 at day 28</td>
<td>0.25</td>
<td>0.02 – 0.73</td>
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<td>0.03</td>
<td></td>
</tr>
<tr>
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<td>0.56 – 2.28</td>
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<td>0.3 – 2.06</td>
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<tr>
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<td>0.02</td>
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<tr>
<td>ADAMTS5</td>
<td>10 ng/ml rhBMP-7 at day 7</td>
<td>100 ng/ml rhBMP-7 at day 7</td>
<td>-1.96</td>
<td>-2.69 – -0.78</td>
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<td>0.03</td>
<td></td>
</tr>
<tr>
<td>MMP13</td>
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<td>10 ng/ml rhBMP-7 at day 7</td>
<td>-3.62</td>
<td>-6.72 – -0.27</td>
<td>98</td>
<td>0.02</td>
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</tr>
<tr>
<td>TIMP1</td>
<td>- control at day 7</td>
<td>- control at day 7</td>
<td>-2.81</td>
<td>-3.86 – -1.16</td>
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<td>CASP3</td>
<td>control at day 28</td>
<td>control at day 28</td>
<td>-2.57</td>
<td>-3.34 – -0.91</td>
<td>99</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control at day 28</td>
<td>10 ng/ml rhBMP-7 at day 28</td>
<td>-1.84</td>
<td>-3.16 – -0.00</td>
<td>96</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>BAX/BCL2</td>
<td>control at day 28</td>
<td>control at day 7</td>
<td>-1.16</td>
<td>-1.49 – -0.27</td>
<td>98</td>
<td>0.02</td>
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</tr>
<tr>
<td></td>
<td>control at day 7</td>
<td>control at day 7</td>
<td>0.71</td>
<td>0.02 – 1.24</td>
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<td>0.02</td>
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</tbody>
</table>

*Difference in location estimates the median of the difference between condition x and condition y.

ACAN = aggrecan, COL2A1 = collagen type 2, CCND1 = cyclin-D1, ADAMTS5 = a disintegrin and metalloproteinase with thrombospondin motifs 5, MMP13 = matrix metalloproteinase 13, TIMP = tissue inhibitor of metalloproteinase 1, CASP = caspase 3, BAX = B-cell lymphoma 2-associated X, BCL2 = B-cell lymphoma 2
Table 2. Significant differences and confidence intervals of statistical analyses performed on data of the *in vivo* experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition vs</th>
<th>Condition</th>
<th>Hazard ratio (HR)</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP3</td>
<td>NP</td>
<td>AF</td>
<td>2.13</td>
<td>1.02 – 4.44</td>
<td>96</td>
<td>0.03</td>
</tr>
<tr>
<td>BAX/BCL2</td>
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<td>Sham</td>
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<td>1.07 – 12.52</td>
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<td>0.02</td>
</tr>
<tr>
<td></td>
<td>25 μg rhBMP-7</td>
<td>250 μg rhBMP-7</td>
<td>0.26</td>
<td>0.08 – 0.89</td>
<td>99</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Figure 5. Relative gene expression of genes associated with apoptosis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
<th>vs Condition</th>
<th>Hazard ratio (HR)</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1</td>
<td>NP</td>
<td>AF</td>
<td>0.22</td>
<td>0.08 – 0.61</td>
<td>99</td>
<td>0.0001</td>
</tr>
<tr>
<td>COL2A1</td>
<td>NP</td>
<td>AF</td>
<td>2.46</td>
<td>1.07 – 5.65</td>
<td>99</td>
<td>0.006</td>
</tr>
<tr>
<td>GAG/DNA</td>
<td>NP</td>
<td>AF</td>
<td>1.13</td>
<td>0.99 – 1.26</td>
<td>99</td>
<td>&lt;0.0001</td>
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</tbody>
</table>

Supplementary file 4. Relative gene expression of bone morphogenetic protein antagonist noggin (NOG) in intervertebral discs injected with rhBMP-7

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
<th>vs Condition</th>
<th>Hazard ratio (HR)</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOG</td>
<td>25 μg rhBMP-7</td>
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<td>0.02 – 0.63</td>
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<td>0.0009</td>
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<td></td>
<td>25 μg rhBMP-7</td>
<td>Sham</td>
<td>0.15</td>
<td>0.03 – 0.73</td>
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<td>0.002</td>
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*DNA = deoxyribonucleic acid, GAG = glycosaminoglycan, rhBMP-7 = recombinant human bone morphogenetic protein-7, CASP = caspase 3, BAX = B-cell lymphoma 2-associated X, BCL2 = B-cell lymphoma 2, NOG = noggin*
Relative gene expression of BMP antagonist noggin (*NOG*) in canine intervertebral discs injected with rhBMP-7. Significant differences and the corresponding confidence intervals are represented in Additional file 3. **Indicates significant difference at a 99% confidence interval**
Biocompatibility and intradiscal application of a thermoreversible celecoxib-loaded poly-(N-isopropyl-acrylamide) MgFe-layered double hydroxide (pNIPAAM MgFe-LDH) hydrogel in a canine model


Arthritis Research & Therapy (2015) 17:214
Abstract

Introduction
Chronic low back pain due to intervertebral disc (IVD) degeneration is associated with increased levels of inflammatory mediators. Current medical treatment consists of oral anti-inflammatory drugs to alleviate pain. In this study, the efficacy and safety of a novel thermoreversible poly-(N-isopropylacrylamide) MgFe-layered double hydroxide (pNIPAAM MgFe-LDH) hydrogel was evaluated for intradiscal controlled delivery of the selective cyclooxygenase (COX) 2 inhibitor and anti-inflammatory drug celecoxib (CXB).

Methods
Degradation, release behavior, and the ability of a CXB-loaded pNIPAAM MgFe-LDH hydrogel to suppress prostaglandin E_2 (PGE_2) levels in a controlled manner in the presence of a pro-inflammatory stimulus (TNF-\(\alpha\)) were evaluated in vitro. Biocompatibility was evaluated histologically after subcutaneous injection in mice. Safety of intradiscal application of the loaded and unloaded hydrogels was studied in a canine model of spontaneous mild IVD degeneration by histological, biomolecular, and biochemical evaluation. After the hydrogel was shown to be biocompatible and safe, an in vivo dose-response study was performed in order to determine safety and efficacy of the pNIPAAM MgFe-LDH hydrogel for intradiscal controlled delivery of CXB.

Results
CXB release correlated to hydrogel degradation in vitro. Furthermore, controlled release from CXB-loaded hydrogels was demonstrated to suppress PGE_2 levels in the presence of TNF-\(\alpha\). The hydrogel was shown to exhibit a good biocompatibility upon subcutaneous injection in mice. Upon intradiscal injection in a canine model, the hydrogel exhibited excellent biocompatibility based on histological evaluation of the treated IVDs. Gene expression and biochemical analyses supported the finding that no substantial negative effects of the hydrogel were observed. Safety of application was further confirmed by the absence of clinical symptoms, IVD herniation or progression of degeneration. Controlled release of CXB resulted in a non-significant maximal inhibition (~35%) of PGE_2 levels in the mildly degenerated canine IVDs.

Conclusion
In conclusion, this study showed biocompatibility and safe intradiscal application of an MgFe LDH-pNIPAAM hydrogel. Controlled release of CXB resulted in only limited inhibition of PGE_2 in this model with mild IVD degeneration, and further studies should concentrate on application of controlled release from this type of hydrogel in animal models with more severe IVD degeneration.
Intradiscal application of a celecoxib-loaded pNIPAAM MgFe-LDH hydrogel

Introduction

Chronic low back pain is a debilitating disorder associated with intervertebral disc (IVD) degeneration. As the exact pathogenesis is still poorly understood, current surgical and medical treatments aim at alleviating symptoms. Inhibiting or reversing the degenerative process by using advanced methods like cell and tissue engineering are in development, but are not clinically applicable thus far. In degenerative disc diseases, the specific composition of the nucleus pulposus (NP) and annulus fibrosus (AF) is disturbed, since the delicate equilibrium shifts toward the catabolic pathways. In the NP this results in a change from an extracellular matrix rich in proteoglycans and type II collagen, to a tissue containing mainly type I collagen, and in the AF in a loss of lamellar organization. The loss of proteoglycans causes a decrease in the water binding capacity of the NP and together with the changes in the AF, compromises the structural functionality of the IVD.

A variety of inflammatory mediators has been investigated for their role in the catabolic processes of IVD degeneration; targeting inflammation is one of the emerging treatment strategies of chronic low back pain and IVD degeneration. Herniated degenerative disc tissue has been shown to spontaneously produce increased amounts of matrix metalloproteinases (MMPs), nitric oxide, prostaglandin E2 (PGE2) and interleukin 6 (IL-6), and to express interleukin 1 (IL-1), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α). TNF-α and IL-1 upregulate expression of matrix degrading enzymes by NP cells. Furthermore, elevated levels of IL-1 and PGE2 have been associated with aging and degeneration of the IVD. In the NP, PGE2 negatively affects matrix integrity by inhibiting proteoglycan synthesis, possibly mediated by a decrease in insulin growth factor 1 and an increase in matrix degrading enzymes.

PGE2 is a well-known prostanoid and plays an important regulatory role in physiological as well as pathological processes like intervertebral disc degeneration. It is synthesized by two cyclooxygenase (COX) isoforms, COX-1 and COX-2, by conversion of arachidonic acid into PGH2 and isomerization of PGH2 to PGE2 by prostaglandin E synthases (PTGES). COX-1 is constitutively expressed in most tissues and is associated with the production of baseline PGE2 levels important for homeostasis. In contrast, COX-2 expression is highly restricted under physiological conditions, but can be rapidly induced in response to inflammatory stimuli and is therefore believed to play an important role in the PGE2 production involved in degenerative processes. Selective COX-2 inhibitors have been developed to reduce PGE2 production via this pathway. In several clinical trials the efficacy of COX-2 inhibitors in patients with low back pain has been established. However, their widespread application is hampered by severe side effects, such as cardiotoxicity. Although these inhibitors can be effectively introduced into the avascular IVD by intradiscal injection, they would achieve only short-lived clinical effects. Delivering drugs
by using controlled release systems, e.g. hydrogels, would be a more attractive alternative to bolus injections, as a higher loading dose and long term delivery can be accomplished by a minimum of intradiscal interventions.\textsuperscript{15, 16}

Temperature sensitive poly-(N-isopropylacrylamide) (pNIPAAM)-based hydrogels have been extensively used in the field of controlled release.\textsuperscript{17} These gels could be particularly suitable for intradiscal injection as they are liquid at room temperature, and hence injectable through small diameter needles and form a solid gel at 37°C, preventing leakage of injected materials from the IVD.\textsuperscript{18} In this study a hybrid thermoreversible biodegradable hydrogel served as a controlled release platform for the specific COX-2 inhibitor celecoxib (CXB). This release system consists of lower critical solution temperature (LCST) polymers with a low molecular weight, based on poly-(N-isopropylacrylamide) (pNIPAAM) with a sulfonate endgroup, ionically linked to a network of biodegradable platelet type MgFe layered double hydroxide (LDH) nanoparticles. LDH particles possess a positive surface charge, that can interact with the negatively charged LCST polymers. At room temperature these hybrid structures are simply made by mixing the polymer with the MgFe-LDHs and subsequent dispersion in water. This results in a solution of low viscosity that can easily be injected into the NP via a 29G needle. At body temperature (37 °C), physical entanglements are formed due to hydrophobic interactions between the LCST polymers, resulting in the formation of a hybrid network, as polymers are linked to the MgFe-LDH particles. Furthermore, the easily ionizable carboxylic groups of CXB can interact with the biodegradable LDHs, which makes this unique for drug release.

We hypothesize that pNIPAAM MgFe-LDH hydrogels are suitable vehicles for delivering a COX-2 inhibitor into the IVD, to reduce intradiscal PGE\textsubscript{2} levels over time in a dog model with spontaneous IVD degeneration, showing pathophysiological aspects similar to those in man.\textsuperscript{19, 20}

**Materials and methods**

After synthesis, preparation, and rheological analysis of the hydrogel, the degradation and release behavior of the CXB-loaded hydrogel was evaluated. Furthermore, in an *in vitro* model in the presence of a pro-inflammatory stimulus (TNF-\(\alpha\)) the ability of the CXB-loaded hydrogels to suppress PGE\textsubscript{2} levels in a sustained manner was evaluated. Thereafter, biocompatibility upon subcutaneous injection was studied in mice. Safety of intradiscal application of the loaded and unloaded hydrogel was studied in a canine model of spontaneous mild IVD degeneration. After the hydrogel was shown to be biocompatible and safe, a follow up dose-response *in vivo* study was performed in order to determine safety and efficacy of the pNIPAAM MgFe-LDH hydrogel for intradiscal controlled delivery of CXB.
Synthesis and preparation of poly-(N-isopropylacrylamide) (pNIPAAM) MgFe-LDH hydrogels
The poly-(N-isopropylacrylamide) (pNIPAAM) polymer with sulfonate endgroup was synthesized as reported previously\textsuperscript{21} and the modified synthesis is described in detail in Additional file 1. To formulate the hydrogel, the pNIPAAM polymer was added to the LDH suspension in a vial and subsequently placed on a tube roller mixer for 48 hours at room temperature and sterilized by gamma radiation (25 kGy, Isotron BV, Ede). The final hydrogel contained 16 wt% pNIPAAM, 3.3 wt% MgFe LDH and water.

Rheological analysis of the pNIPAAM MgFe-LDH hydrogels
The viscoelastic properties of the unloaded pNIPAAM MgFe LDHs were determined by using an Anton Paar MCR301 rheometer (St. Albans, UK) with an oscillatory parallel plate geometry (50mm diameter) with a constant strain of $\gamma = 0.5\%$ at a frequency of $f = 1$ Hz. Temperature was increased from 22 °C to 37 °C at a rate of 15 °C/min. This heating rate was chosen based on calculating the minimum rate of heat transfer based on estimating the energy needed to heat up the hydrogel by taking into account, the surface of the hydrogel, the minimum temperature difference between the LCST and body temperature. LCST is the critical temperature above which the hydrogel undergoes a phase transition from a soluble to an insoluble state. This estimation is described in detail in Additional file 1. The gelling was recorded by measuring the complex shear modulus $|G^*|$ which is a common parameter to determine the strength of a viscoelastic material like a hydrogel. The complex shear modulus $|G^*|$ is correlated to the storage modulus ($G'$) and loss modulus ($G''$). The storage modulus is a measure of the deformation energy stored in the sample during the shear process (elastic behavior), whereas the loss modulus is a measure of the energy dissipated in the sample during the shear process (viscous behavior), and is lost to the sample afterwards (viscous behavior). The relation between these parameters is the following:

$$|G^*| = \sqrt{(G')^2 + (G'')^2}$$

Hydrogel samples were placed on the lower plate, and the upper plate was lowered to a 0.5mm gap. The configuration of the rheological setup is shown in Figure 1A. The viscoelastic properties of the loaded hydrogel were not determined. The CXB concentrations were as low as $10^{-6}$ M to $10^{-4}$M, i.e. 0.38 -38 mg of celecoxib per liter, or 0.0038 - 3.8*10^{-5} wt%, and were not influencing the rheological properties.
Degradation and release behavior of CXB-loaded pNIPAAM MgFe-LDH hydrogels

*In vitro*, the controlled release of CXB from hydrogels was measured in PBS (phosphate buffered saline pH 7.4, 44 mM Na$_2$HPO$_4$, 9 mM NaH$_2$PO$_4$, 72 mM NaCl, 0.02% wt Na$_3$) and 0.2% Tween 80° (polyoxyethylenesorbitan monooleate; Sigma-Aldrich). Tween 80° was added to the buffer in order to increase the solubility of CXB$^{22}$ and thereby simulate the *in vivo* situation. CXB-loaded pNIPAAM MgFe-LDH suspension was prepared by adding 6 or 10 mg/ml of CXB to the dispersion and stirring with a stirring bar over the weekend. A volume of 1 ml of CXB-loaded pNIPAAM MgFe-LDH suspension was pipetted into a vial and placed for 30 minutes at 37°C to ensure gelation of the hydrogel, and afterwards covered with 14 ml warm (37°C) PBS/Tween 80° solution. The release experiment was performed at 37°C At day 1, 2, 5, 8, 15, 22, and 31, 12 ml of the buffer solution was removed in order to analyze CXB and Mg concentrations and 12 ml of fresh buffer was added.

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**Figure 1.** Rheological setup and results of the complex shear modulus $|G^*|$ of the poly(N-isopropylacrylamide) (pNIPAAM) MgFe-layered double hydroxide (LDH) hydrogel. A. Configuration of the rheological setup to measure $|G^*|$ of the pNIPAAM MgFe-LDH hydrogel. B. $|G^*|$ of the pNIPAAM MgFe-LDH hydrogel as a function of temperature. The dashed red line connects the time point where the $|G^*|$ starts to increase to the temperature curve, and indicates a lower critical solution temperature (LCST) of 32°C. NS = non-sterilized, AS = after sterilization, ASC = after sterilization separate components.
CXB concentrations were determined in a volume of 100 μl by using ultra performance liquid chromatography (UPLC) as described in detail recently by Petit et al.\textsuperscript{23} In vitro degradation was determined by measuring the relative cumulative release of Mg into the medium. Mg concentrations were determined by using a Prodigy High Dispersion Inductively Coupled Optical Emission Spectrometry (ICP-OES) system (Leeman, Illinois, USA). Standards were prepared by using multi-element (23 elements in diluted nitric acid) standard solution IV (1000 mg/l) (Merck, Darmstadt, Germany). A volume of 0.5 ml of the solutions from the degradation experiment was diluted in 100 ml aqueous 1N HNO₃, and subsequently diluted again tenfold in 1N HNO₃. The effects of CXB loading (10 mg/ml versus 6 mg/ml), LDH content (single versus double) and type of LDH (Mg₃Fe versus Mg₂.5Fe) of the gels on release behavior and in vitro degradation were also investigated, as well as CXB solubility effects by using a buffer containing PBS and 0.2% or 2% Tween 80°.

**Controlled release of CXB in vitro**

The balance between anabolic and catabolic pathways in articular chondrocytes as well as NP cells can be directed towards catabolism by TNF-α.\textsuperscript{24, 25} Bovine articular chondrocytes were used in the in vitro experiments, as they were more easily available in our laboratory. Articular chondrocytes were isolated from bovine carpometacarpal joints by enzymatic digestion overnight with 2 mg/ml collagenase A (Roche, Mannheim, Germany) at 37°C. Chondrocytes were seeded at 5 x 10⁶ cells/ml density (P0) into cylindrical (diameter and height 6 mm) 2% agarose (Type VII, Sigma Aldrich, Zwijndrecht, The Netherlands) constructs and left to gel at room temperature. The constructs were then cultured in 12-wells plates in high glucose Dulbecco’s modified Eagle’s medium (hgDMEM; Gibco, Life Technologies Europe, Bleiswijk, The Netherlands) with 20% fetal bovine serum (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 0.1% amphotericin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), 1% Pen-Strep (Biochrom GmbH, Berlin, Germany), 1% non-essential amino acids (Lonza, Basel, Switzerland) 1% essential amino acids (Lonza), and 50 mg/ml ascorbate 2-phosphate (Biochrom). During the first 5 days of culturing, these constructs were stimulated with 10 ng/ml TNF-α to induce an inflammatory response reflected by elevated PGE₂ levels (Figure 2A). A concentration of 1 μM CXB has been described to effectively lower PGE₂ levels in osteoarthritic chondrocytes and corresponds with mean pharmacological plasma levels.\textsuperscript{26} CXB was dispersed in the pNIPAAm MgFe-LDH mixture at a concentration of 0.1 mg/ml, aiming to establish a concentration of approximately 1 μM CXB per culture medium renewal over the 28-day culture period. Controlled release of the CXB is achieved by dissolution of the CXB crystals present a depot within the hydrogel and diffusion of the solubilized CXB. A volume of 100 μl of the hydrogel suspension was pipetted at the bottom of a 12-wells plate and placed in an incubator at 37°C to ensure gelation of the hydrogels. Subsequently, defined as day 0 of the experiment, cell constructs and culture medium
were added to the 12-wells plate. For the “bolus injection” of CXB, only cell constructs were placed on the bottom of the well, and CXB was added to the medium every 2 days, starting at day 0, at a concentration of 1 μM. Media were renewed every 2-3 days, collected on days 0, 2, 7, 9, 11, 14, 21, and 28 and stored at -80°C for analysis of CXB content. For the in vivo experiments based on formulation of higher doses of CXB, in vitro experiments were also carried out with a higher dosage of CXB to evaluate release profiles at higher dosing. To this end, 1 mg of CXB-loaded per ml of hydrogel, aiming to establish a concentration of 10 μM CXB per culture medium renewal was used. Conditioned media were analyzed for CXB content and PGE₂ levels. Inhibition of COX-2 activity was determined by measuring PGE₂ in culture medium. A colorimetric competitive enzyme immunoassay kit (PGE₂ EIA kit, ENZO Life Sciences BVBA, Antwerp, Belgium) was used to determine PGE₂ levels in culture medium according to the manufacturer’s instructions.

**In vivo biocompatibility in mice after subcutaneous implantation**

All animal procedures were approved and performed in accordance with the guidelines set by the Animal Experiments Committee (DEC) of Utrecht University (experiment numbers: DEC 2010.III.03.046; DEC 2012.III.05.046 and DEC 2013.III.02.017). Six healthy female adult (8 – 10 wks) BALB/c mice, (Harlan-Olac, Bicester, United Kingdom) were used for testing biocompatibility and biosafety of the pNIPAAM MgFe-LDH polymer hydrogel and 7 other biomaterials. Four different biomaterials were injected at least 1 cm apart from each other into the dorsal subcutaneous tissue of each mouse in a randomized fashion. Buprenorphine 100 μg/kg was given intraperitoneally (i.p.) as premedication and analgesic and subsequently all animals were anesthetized with isoflurane via an induction mask (vaporizer setting 2.5 %) in a 1:1 oxygen:air mixture. A blood sample was drawn to perform a white blood cell count and differentiation at day 0, to rule out systemic inflammation. A volume of 200 μl of each biomaterial was injected subcutaneously with a 27G needle under sterile conditions. PBS (200 μl) served as a control. All injection sites were marked with a waterproof marker. Immediately after injection, Dermabond® (Ethicon, Cornelia, USA) was applied to the injection site to prevent leakage and the injection site was heated by an infrared lamp for 1 minute. Mice were monitored daily for signs of distress or pain (e.g. lethargy, weight loss, automutilation, and abnormal posture) and injection sites were monitored for inflammation (e.g. swelling, redness, pain, and heat). Three animals were sacrificed 7 days after injection, and three after 28 days. At the end of the experimental period, animals were anesthetized with isoflurane, blood was collected by cardiac puncture for white blood cell count and differentiation, and euthanasia was performed by cervical dislocation. The injection sites were removed for histological analysis. Tissues were fixated in a 4% neutral buffered formaldehyde solution (Klinipath B.V., Duiven, The Netherlands) and after fixation routinely embedded in paraffin. Sections of 4 μm were stained with hematoxylin and eosin. Infiltration of
inflammatory cells, giant cells, necrosis, neovascularization, fatty infiltration, and the encapsulation of the biomaterial by a fibrotic capsule were histologically assessed as parameters for a biological response at the application site, at 7 and 28 days by a blinded board-certified veterinary pathologist (GG) and the principal investigator (NW) using an Olympus BX41 microscope.

**Intradiscal application of CXB-loaded pNIPAAM MgFe-LDH hydrogels in laboratory beagle dogs**

Data from two *in vivo* studies in beagle dogs were combined and analyzed. Both studies were set up as randomized block designs. In the first study CXB-loaded (7.7 μM) and unloaded pNIPAAM MgFe-LDH hydrogels, a bolus injection of CXB (7.7 μM) and 0.9% NaCl were intradiscally injected. In other levels two other materials irrelevant to this study were injected. The second study served as a dose response study, including a 10- and 100-fold higher dosage of CXB (77 μM and 770 μM) in addition to the 7.7 μM dose. For preparation of the CXB-loaded hydrogels, CXB was prepared from a CXB stock solution in ethanol (60 μg/ml) by sterile filtration. Water was added to this ethanolic solution of CXB to obtain a dispersion with small CXB crystals (Ø approximately 1 μm). This dispersion was freeze-dried overnight and the pNIPAAM MgFe-LDH mixture was added and incubated overnight on a tube roller mixer at room temperature.

In total eighteen intact female beagle dogs (Harlan, Gannat, France) with a median age of 1.7 years (range 1.3 – 1.8 years) and a median weight of 8.4 kg (range 6.2 – 13.8 kg) were used. Nine dogs with a median age of 1.6 years (range 1.3 – 1.8 years) and a median weight of 8.2 kg (range 6.2 – 11 kg) were used in the first study. Nine dogs with a median age of 1.7 years (range 1.6 – 1.8 years) and a median weight of 9.3 kg (range 8.3 – 13.8 kg) were used in the second study. All dogs underwent general, orthopedic, and neurologic examination by a board-certified veterinary surgeon (BM).

**Surgical procedure**

To determine the grade of degeneration of the IVDs prior to surgery, MR images of the lumbar vertebral column were obtained in fully anesthetized dogs. A blood sample was drawn from the jugular vein to assess white blood cell count and differentiation, to exclude systemic inflammation. Dogs were placed in a dorsal recumbent position and throughout the complete scan protocol heart rate, respiration rate, temperature, carbon dioxide, and oxygen levels were monitored. The MRI was performed using a 0.2 Tesla open magnet (Magnetom Open Viva, Siemens AG, Munich, Germany). All lumbar IVDs were assessed according to the Pfirrmann score by a veterinary radiologist on sagittal T2-weighted FSE images (3.0 mm slices, TR 4455 ms, TE 117 ms).

Only lumbar IVDs with a Pfirrmann score II were included for injection.
The anesthesia protocol during surgery was similar to the one used during MRI scanning. Analgesia was provided by a combination of fentanyl (loading dose 10 μg/kg, 15-20 μg/kg/hr continuous rate infusion, CRI) and ketamine (0.5 mg/kg loading dose, 10 μg/kg/min CRI) intravenous (IV). Throughout the complete procedure heart rate, respiration, temperature, carbon dioxide, oxygen levels, and blood pressure (noninvasive) were monitored. Surgical sites were prepared according to standard protocol. A detailed description of the surgical procedure has been described previously. Briefly, dogs were positioned in right recumbence to expose and inject the T13-L1 until L6-L7 via a left lateral approach. To diminish injury of the m. iliopsoas and sciatic nerve traction injury, the surgical approach in the second study was adjusted and L6-L7 and L7-S1 were injected via a dorsal approach, while the dogs were positioned in ventral recumbence. In the first study a 100 μl syringe (7638-01 Model 710 RN, Hamilton Company USA, Reno, Nevada, USA), and in the second study a 100 μl gastight syringe (7656-01 Model 1710 RN) with a 29G needle (25 mm, 12° beveled point; Hamilton Company USA, Reno, Nevada, USA) was used to inject 30 μl of the earlier mentioned compounds through the AF into the NP. The smallest possible needle diameter was chosen to minimize injury to the treated IVDs. Wound closure was performed according to standard protocol. Postoperative pain management in all dogs consisted of methadone 0.3 mg/kg intramuscular (IM) six times a day during the first 24 hours postoperatively and buprenorphine 20 μg/kg IM four times a day and/or tramadol 2-5 mg/kg orally three times a day the following 7 days. All dogs were treated postoperatively with antibiotics (amoxicillin/clavulanic acid 12.5 mg/kg orally twice a day) during 5 days. Dogs were monitored daily throughout the study by a veterinarian to assess pain symptoms according to the short form of the Glasgow composite pain scale. Dogs that showed signs of pain, received tramadol and/or buprenorphine and/or gabapentin (5 mg/kg orally twice a day). Furthermore, animals were monitored daily by a veterinarian for clinical signs of illness, neurologic deficits and lameness.

**Injected substances**

In the first study spontaneously degenerated IVDs (Pfirmann grade 2) of the dogs were injected with a volume of 30 μl of NaCl 0.9% (sham), a bolus of CXB (7.7 μM), a CXB-loaded (7.7 μM) and an unloaded pNIPAAM MgFe-LDH hydrogel. Based on studies in cadaveric spines (unpublished data, N. Willems and B.P. Meij) a volume of 30 μl could be injected into the NP without substantial resistance. The volume of 30 μl contained 7.7 μM CXB, to achieve a final concentration of 1 μM (=7.7*10^{-6} * (30 μl gel/230 μl NP volume plus gel)) for the bolus of CXB, in the canine NP of beagle laboratory dogs with a mean weight of 8 – 9 kg and taking into account the volume of the nucleus (200 μl). All substances were injected into the IVDs in the T12-L6 spinal segment in a randomized fashion, except for the sham treatment (NaCl 0.9%), which was injected into T12-T13. An interim statistical
Intradiscal application of a celecoxib-loaded pNIPAAM MgFe-LDH hydrogel analysis was performed after the first study to evaluate treatments and study design. Results were used to perform a new power analysis and to adapt the study design of the second study. In the second study, all substances were administered in a random order within each animal and IVDs of the T12-S1 spinal segment were injected with NaCl 0.9%, a bolus of CXB (7.7 μM), CXB-loaded (7.7 μM, 77 μM and 770 μM) hydrogels, and an unloaded pNIPAAM MgFe-LDH hydrogel. IVDs adjacent to those injected with hydrogel loaded with the highest dose of CXB (770 μM) remained untreated.

Post-mortem collection of materials
Dogs were euthanized 4 weeks post-injection. First, they were sedated with dexmedetomidine 0.04 mg/kg IV, followed by pentobarbital 200 mg/kg IV. Immediately after euthanasia, the vertebral column (T12 – S1) was harvested by using an electric multipurpose saw (Bosch, Stuttgart, Germany). All muscles were removed and the vertebrae were transected transversely with a band saw (EXAKT tape saw, EXAKT Advanced Technologies GmbH, Norderstedt, Germany), resulting in nine spinal units (endplate – IVD – endplate). These units were then transected sagittally by using a diamond band pathology saw (EXAKT 312 saw; EXAKT diamond cutting band 0.1 mm D64; EXAKT Advanced Technologies GmbH, Norderstedt, Germany), generating two identical parts. One half was resected with a surgical knife by removing the endplate and the vertebra attached to it on one side, and the remaining IVD tissue was snap frozen in liquid nitrogen stored at -80°C for biochemical and biomolecular analyses. The other half was photographed (Olympus VR-340, Hamburg, Germany) for macroscopic evaluation of the IVD (Thompson score, see below) and stored for 14 days in 50 ml of 4% buffered formaldehyde at 4°C for histological analyses.

Histology, COX-2 immunohistochemistry, and TUNEL assay
Samples were decalcified in 35% formic acid and 6.8% sodium formate in a microwave oven (Milestone Microwave Laboratory Systems, Italy) overnight at 37°C, during 7 nights and embedded in paraffin. Five μm thick sections were stained with hematoxylin and eosin and with picrosirius red/alcan blue and evaluated according to a grading scheme according to Bergknut et al. Histological slides were scored blinded and in random order by two independent investigators (NW, AT) using an Olympus BX41 microscope. In case of doubt, samples were also scored by a board-certified veterinary pathologist (GG). All photographs of the macroscopy of the IVD segments were evaluated by two independent blinded investigators (NW, AT) according to the Thompson grading scheme, which has been validated in dogs.

Immunohistochemistry for COX-2 was performed on 5 μm sections mounted on KP plus glass slides. After deparaffinization and rehydration sections were treated with Dual
Endogenous Enzyme Block (Dako S2003, California, USA) for 10 min at room temperature to block nonspecific endogenous peroxidase, followed by 2 washing steps of each 5 min with tris buffer saline 1% Tween 20® (TBS-T). Sections were treated with TBS bovine serum albumin (BSA) 5% solution to block non-specific binding for 60 minutes at room temperature, were carefully rinsed and subsequently incubated with a primary mouse anti-human monoclonal COX-2 antibody (Cayman, Ann Arbor, USA) diluted 1:50 in TBS-BSA 5% overnight at 4°C. The following day sections were incubated with peroxidase-labelled polymer (Envision anti-mouse (K4001, Dako). Antibody binding was visualized by using diaminobenzidine (DAB; Dako). Sections were counterstained with hematoxylin solution (Hematoxylin QS, Vector, Peterborough, UK) and mounted in permanent mounting medium.

A commercial available terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL; Millipore, Darmstadt, Germany) assay was used according to the manufacturer’s instructions to determine apoptosis. The percentage of COX-2 positive and TUNEL positive chondrocytes over the total number of cells was determined by manual counting in the NP, and in the ventral (VAF) and dorsal AF (DAF), by two blinded independent investigators (NW, SP).

**Biomolecular and biochemical analyses**

Cryosections (60 μm) of the spinal units were cut with a cryostat (Leica CM1800 cryostat, Leica Microsystems Inc., Bannockburn, USA) and collected on RNA-se free glass slides. The NP and AF tissues were separated and half of the slides were collected in respectively 400 μl and 750 μl Ambion® KDalert™ lysis buffer solution (Life Technologies, Bleiswijk, Netherlands) in the first study, and in cOmplete lysis M EDTA-free buffer (Roche Diagnostics Nederland B.V., Almere, The Netherlands) in the second study and stored at -80°C until biochemical analyses were performed. The other half was collected in 300μl RLT buffer containing 1% β-mercapto-ethanol (Qiagen, Venlo, The Netherlands) and stored at -80°C until biomolecular analyses were performed.

Quantitative PCR (qPCR) was performed to assess the effects of (controlled release of) CXB at gene expression levels of the NP with regards to: 1) ECM anabolism: aggrecan (ACAN), collagen type II (COL2A1), collagen type I (COL1A1); 2) ECM catabolism (a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinase 1 (TIMP1); 3) inflammation: tumor necrosis factor alpha (TNFA), interleukin-1β (IL1B), interleukin-6 (IL6) and interleukin-10 (IL10); 4) COX pathway and PGE2 synthesis: prostaglandin E synthase 1 (PTGES1), prostaglandin E synthase 2 (PTGES2), cyclooxygenase 1 (COX1), and cyclooxygenase 2 (COX2); 5) notochordal markers: brachyury (T), cytokeratin-8 (CK8), cytokeratin-18 (CK18);
6) the indirect effect of CXB on Wnt signaling pathway: axin-2 (AXIN2), c-Myc (c-Myc) and cyclin-D1 (CCND1) and 7) apoptosis: caveolin-1 (CAV1), caspase 3 (CASP3), fas ligand (FasL) and Bcl-2 (BCL2). The primer pairs used for qPCR are given in Additional file 2. The RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, The Netherlands) was used to isolate total RNA. To maximize RNA yield, the incubation period with proteinase K was reduced to five minutes. After on-column DNase-I digestion (Qiagen RNase-free DNase kit) RNA was quantified by using a NanoDrop 1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). cDNA was synthesized from 20 ng total RNA in a total volume of 15 µl using the iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands). qPCR was performed in duplicate using an iCycler CFX384 Touch thermal cycler, and IQ SYBRGreen Super mix (Bio-Rad). All dog-specific primers were designed in-house using Perlprimer except for MMP13. Primer specificity was evaluated with BLAST, and the designed amplicon was tested for secondary structures using MFold. Primers were purchased from Eurogentec, Maastricht, The Netherlands. Amplification efficiencies ranged from 80% to 115%. Relative expression levels were determined by normalizing the Ct value of each target gene by the mean Ct value of 3 reference genes, i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S19 (RPS19), and TATA-binding protein (TBP).

To measure glycosaminoglycan (GAG) and DNA in content in the NP and AF, samples in cOmplete lysis M EDTA-free buffer were homogenized in a TissueLyser II (Qiagen) for 2x 30 s at 20 Hz. The supernatant and pellet of each NP and AF were digested overnight in a papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 60°C. GAG content was quantified by using a 1,9-dimethylmethylene blue assay. The Quant-iT™ dsDNA Broad-Range assay kit in combination with a Qubit™ fluorometer (Invitrogen, Carlsbad, USA) was used in accordance with the manufacturer’s instructions to determine DNA content in the papain digested NP and AF supernatant and pellets. DNA content in the supernatants of the NP and AF were negligible and therefore not included in the total content.

PGE2 levels were measured with the same colorimetric competitive enzyme immunoassay kit (PGE2 high sensitivity EIA kit, ENZO Life Sciences BVBA) that was used for the in vitro experiments. Both buffers that were used to lyse tissue, were validated and standards were diluted in the same lysis buffer as the samples, which did not show strong interference with the performance of the kit. Total GAG content and PGE2 levels were normalized for DNA content in the pellet and were measured in the NP as well as the AF.

Statistical analyses
Power analyses were performed prior to both in vivo studies by using free software (http://www.stat.uiowa.edu/~rlenth/Power), and are described in detail in Additional file
4, PGE2/DNA in the NP was considered to be the main read-out parameter. Biochemical and biomolecular data were analyzed by using R statistical software, package 2.15.2. A linear mixed effect model was used to analyze the effect of the injected treatments. Factors incorporated into the model as a fixed effect were ‘treatment’ (NaCl, CXB 7.7 μM, CR, CR+7.7 μM, CR+77 μM, CR+770 μM), ‘tissue’ (NP and AF), and their interaction. Random effects ‘dog’ (dog 1-18) and ‘study’ (study 1 and 2) were incorporated to capture the correlation between multiple measurements within one dog. Residual plots and quantile-quantile(QQ)-plots were used to check for possible violations of normality assumptions. In case of violation, data were logarithmically transformed. The Cox proportional hazards regression model was used to estimate the effect of the injected treatments on gene expression levels. Calculations were performed on Ct values for each target gene and the mean Ct value of 3 reference genes was incorporated into the model as a covariate. If proportional hazard assumptions were violated, the ratio of the Ct values for each target gene to the mean Ct value of the reference genes was used for analysis. Ct values ≥ 40 were right censored. Regression coefficients were estimated by the maximum likelihood method. Model selection was based on the lowest Akaike Information Criterion. Confidence intervals were calculated and stated at the 99% confidence level to correct for multiple comparisons. Differences between treatments were considered significant if the confidence interval did not include 0, whereas hazard ratios were considered significant if the confidence interval did not include 1.

Results

Rheological properties and handling of the pNIPAAM MgFe-LDH hydrogels
At low temperatures (22 °C) the complex modulus |G*| = 10Pa and at high temperatures (37 °C) the complex modulus |G*| = 2kPa. Typical mechanical properties of a NP are in the range of 7-21 kPa. However, this hydrogel was not intended to be used as a replacement for the NP and therefore no load-bearing properties were needed. The slightly lower modulus of the hydrogel was sufficient for the purpose of controlled drug release, and for the intradiscal injection of a small volume, without increasing intradiscal pressure with inherent effects on homeostasis of the resident cells. Sterilization with gamma radiation did not have a significant effect on the rheological properties of the gel (Figure 1B). The viscous hydrogel transitioned from a low-viscous state to a stable hydrogel state within 10 seconds due to the hydrophobic interactions between the isopropyl groups of pNIPAAM upon increasing the temperature above its Lower Critical Solution Temperature (LCST) 32 °C (Figure 3A and 3B). The low viscosity solution could easily be injected at room temperature via a 29G needle.
Degradation and controlled release of CXB from pNIPAAM MgFe-LDH hydrogels in vitro

A cumulative release of 14% CXB from hydrogels loaded with 10 mg/ml CXB was shown after 31 days, whereas a cumulative release of 11% Mg was detected, the former indicating that CXB is still present in the hydrogel and controlled release of CXB is most probably accomplished over more than 31 days. The release of CXB showed a similar pattern as dissolution of the MgFe LDH particles in PBS/0.2% Tween® (Additional file 1). An increase in the concentration of Tween® is known to accelerate gel degradation and increase CXB solubility and resulted in a 3-fold higher Mg release and a 2- to 3-fold higher release of CXB (Additional file 1).22 Neither an increase in the amount of LDH particles, nor the charge of Mg affected the CXB release profile. Furthermore, a 1.5-fold increase in the cumulative release of CXB could be detected in gels with a 6 mg/ml loading dose of CXB compared with a 10 mg/ml loading dose. However, the absolute amount of CXB was comparable (20% of 10 mg/ml vs 30% of 6 mg/ml)(Additional file 1). Hydrogels with higher amounts of LDH particles showed a lower amount of cumulative Mg release (Additional file 1). Degradation of pNIPAAM MgFe-LDH hydrogels and controlled release of CXB in vitro are illustrated in the figures depicted in Additional file 1.

Figure 3. A. Schematic diagram of the formation of stable hybrid hydrogels. Positively charged layered double hydroxides (LDH) in a poly(N-isopropylacrylamide) (pNIPAAM) solution (pNIPAAM chain) transit from a low-viscous state to a stable hydrogel due to hydrophobic interactions between the isopropyl groups of pNIPAAM upon increasing temperature. The celecoxib (CXB) is present in the hydrogel in small crystals, forming a depot, from which dissolution and diffusion takes place. B. At 37°C gelation occurs within 10 seconds. C. One month after intradiscal injection the pNIPAAM MgFe-LDH hydrogel (white arrow) is visible in the nucleus pulposus (**). The annulus fibrosus is indicated with a black arrow.
Agarose–cell constructs incubated with 10 μM CXB bolus for 2 consecutive days apparently had taken up CXB by diffusion, leading to detectable amounts of CXB in the medium up to 7 days, which then dropped to zero (Figure 2B). CXB released from the 0.1 mg/ml loaded hydrogels into the culture medium ranged from 1.1 – 4.2 μM (Additional file 3). CXB released from hydrogels loaded with 1 mg/ml CXB resulted in CXB concentrations in the medium ranging from 3 – 15.9 μM. To determine the activity of the released CXB, cells in the constructs were stimulated by 10 ng/ml TNF-α, which resulted in detectable PGE₂ levels in the culture medium at day 9. Application of the 10 μM bolus of CXB during 2 consecutive days resulted in suppression of PGE₂ levels from day 9 to 11, and PGE₂ levels started to increase afterwards to similar levels as the TNF-α stimulated constructs. In the constructs cultured in the presence of 1 mg/ml CXB-loaded hydrogels, TNF-α induced PGE₂ production was completely inhibited throughout the whole culture period of 28 days (Figure 2C).

Figure 2. In vitro controlled release (CR) of celecoxib (CXB) results into sustained suppression of prostaglandin E₂ (PGE₂) levels in the presence of a pro-inflammatory stimulus. A. Experimental setup to evaluate the controlled release of CXB in vitro. During the first 5 days of culturing, 3D-chondrocyte constructs were stimulated with a pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (10 ng/ml). At day 0 of the experiment, a 10 μM bolus injection of CXB was applied for 2 consecutive days or pNIPAAM MgFe-LDH hydrogels loaded with 1 mg/ml CXB (CR CXB). Media were refreshed every 2-3 days and collected on days 0, 2, 7, 9, 11, 14, 21, and 28. On days 14 and 28 the constructs were evaluated for cell viability. B. Celecoxib (CXB) concentrations (μM) were measured in medium samples after administering the bolus injection of CXB or the pNIPAAM MgFe-LDH hydrogel loaded with 1 mg/ml CXB (CR CXB) C. PGE₂ concentrations (ng/ml) measured in medium samples in the following conditions: unstimulated, stimulated with 10 ng/ml TNF-α (TNF-α stimulated), TNF- α stimulated in the presence of a 10 μM bolus injection of celecoxib (CXB bolus) for 2 consecutive days, or 1 mg/ml CXB-loaded pNIPAAM MgFe-LDH hydrogels (CR+CXB).
**In vivo biocompatibility in mice**

Subcutaneous injection of the different hydrogels showed no adverse local or systemic effects. At 7 days post-injection a fibrous capsule of varying thickness (57 – 76 $\mu$m) was present in all hydrogel-injected tissue samples. At the interface between the hydrogel and this capsule mainly neutrophils and macrophages were present, consistent with an acute/subacute pyogranulomatous reaction (Figure 4). At 28 days post-injection the hydrogel and fibrous capsules were also present. In two samples a decreased thickness of the fibrotic capsules (35 and 40 $\mu$m) was observed, whereas in one sample a thickened capsule (236 $\mu$m) was observed. In all three samples macrophages constituted the predominant cell type in the intermediate layer, consistent with a granulomatous reaction. Some of these macrophages showed marked evidence of phagocytic activity. In two samples injected with PBS (control), a slight increase in macrophages was seen at day 28 compared with day 7. Giant cells, necrosis, neovascularization, and fatty infiltration were not observed at either time point.

**Intradiscal application and controlled release of CXB-loaded hydrogels in laboratory beagle dogs**

*Surgical follow up*

Before surgery a total of 162 IVDs were graded on MR images. 9/162 IVDs were assigned a grade I according to the Pfirrmann system, whereas 153/162 IVDs were assigned a grade II, of which 88 were injected in this study. Six out of nine dogs in the first study were ambulant the day after the injections of the test substances in the IVD and showed a slight reduction in spinal reflexes that recovered within the following 7 days. Three dogs also showed reduced weight bearing of the left hind limb, and received pain medication for a longer period of time. Two dogs that received pain medication for 7 more days, recovered completely. In one of these dogs slight dehiscence of the wound was detected and antibiotics were given for a total of 14 days. In one dog the reduction in spinal reflexes and weight bearing of the left hind limb persisted and this dog was also treated with gabapentin 5 mg/kg p.o. q.12.h. All dogs in the second study showed uneventful recovery from surgery, were ambulant the next day and showed minor reductions in spinal reflexes that recovered within 7 days.
Figure 4. Representative histological images of subcutaneous injection sites in mice (hematoxylin and eosin stain). A-C show the skin and subcutis of a control animal at 7 days revealing the epidermis (arrow a), hair follicles (arrowhead a) and striated muscle (panniculus carnosus; asterisk A-C). The panniculus carnosus (asterisk C) and an occasional mast cell (arrowhead) are visible in the subcutis. D-F show the skin 7 days after injection of the hydrogel. The hydrogel is visible as a grey, granular substance (white arrow E) positioned below the panniculus carnosus (arrowhead E) surrounded by a capsule of loosely arranged fibroblasts (asterisk E). Multifocally infiltrates of eosinophils (white arrow F), neutrophils (arrowhead F) and macrophages (black arrow F) separating the hydrogel from the fibrous capsule. G-I show the skin of a control animal at 28 days after injection without significant pathological changes. J-L depict the histological changes in the subcutis 28 days after injection of the hydrogel with the epidermis and panniculus carnosus indicated by an arrow and arrowhead respectively in J. K shows a more compact fibrous capsule (arrow) compared to the loose capsule seen after 7 days post-injection. The cellular reaction directly surrounding the hydrogel show a more granulomatous nature indicated by the presence of macrophages often containing brown pigment (L).
Intradiscal application of a celecoxib-loaded pNIPAAM MgFe-LDH hydrogel

**IVD integrity**

Post-mortem, Thompson score grade II was assigned to 87/88 IVDs; a grade III was assigned to 1/88 IVDs, which had been injected with the unloaded hydrogel. In 17/52 IVDs injected with the hydrogel, the tan colored hydrogel was visible in the mid-sagittal sections of the NP (Figure 3C). Histological evaluation of the total of 88 IVDs was performed according to the grading scheme according to Bergknut et al.\(^3\) (Figure 6a). Scores ranged from 4 – 14. The median histological grade in the first study was 9.5 (4 – 13) and in the second study 11 (8 – 14). No significant differences were found between the injected treatments. In one of the IVDs injected with the empty hydrogel (level L6-L7), fibrotic tissue was present in the dorsal AF; in another IVD injected with the empty hydrogel (level L7-S1), the central parts of both sides of the EPs were very irregular and clusters of chondrocytes were present in the dorsal AF at both sides of the AF-EP interface. In one of the IVDs injected with NaCl (level T12-T13), acellular material was detected in the ventral AF. At macroscopic examination slight bulging of the ventral AF was noticed.

Relative gene expression levels of \(BCL2\), a regulatory gene of cell death (apoptosis) were significantly downregulated in the CXB-loaded hydrogel compared with the sham \((HR = 8.28, CI 99\% 1.45 – 47.19)\) (Figure 5A). However, gene expression levels of other apoptotic markers, i.e. \(CAV1\), \(CASP3\), and \(FasL\) showed no signs of increased apoptosis in any of the treatments. Percentages of TUNEL-positive cells per total cell count showed no differences between treatments either (median 0%, range 0% - 82%). Furthermore, there were no significant differences in gene expression levels of notochordal cell markers \(T, CK8, CK18\), nor in levels of \(AXIN2\), \(c-Myc\), and \(CCND1\), associated with the Wnt pathway between the treatments.

**Extracellular matrix metabolism**

Relative gene expression of the catabolic gene \(ADAMTS5\) was significantly upregulated in the NP samples treated with the CXB bolus \((HR = 10.35, CI 99\% 1.74 – 61.57)\) and the CXB-loaded hydrogel \((HR = 10.66, CI 99\% 1.70 – 66.67)\) (Figure 5B) compared with the unloaded hydrogel. Gene expression levels of other catabolic \((MMP13)\) and anti-catabolic \((TIMP1)\) genes were not significantly different between treatments (Figure 5B). However, genes associated with extracellular matrix (ECM) components, i.e. \(ACAN, COL2A1, COL1A1\) did not significantly differ between treatments (Figure 5C). These findings were consistent with normalized GAG content (GAG/DNA) in the NP as well as the AF, which did not significantly differ between treatments 4 weeks post-injection. GAG/DNA levels were significantly higher in the NP than in the AF in all treatments \((M = 0.58, SD = 0.03, CI 99\% 0.50 – 0.66)\) (Figure 6B).
Figure 5A-C. Relative gene expression levels per treatment. The NaCl (sham) treatment in nucleus pulposus (NP) tissue is set at 1. A. Relative gene expression levels of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS5) were significantly upregulated in the nucleus pulposus (NP) samples treated with the celecoxib (CXB) bolus (CXB 7.7 μM) and the CXB-loaded poly-(N-isopropylacrylamide) MgFe-layered double hydroxide (LDH) hydrogel (CR + CXB 7.7 μM) compared with the unloaded hydrogel/controlled release system (CR). B. Gene expression levels of aggrecan (ACAN), collagen type 2 alpha 1 (COL2A1), collagen type 1 alpha 1 (COL1A1) did not significantly differ between treatments. C. B-cell lymphoma-2 (BCL2) expression levels were significantly down regulated in the CXB-loaded hydrogel compared with the sham (NaCl). Data are expressed as n-fold changes ± standard deviation. Figure 5D and E. Representative histological images of early degenerated canine NPs injected with NaCl (D) or a 770 μM celecoxib (CXB)-loaded pNIPAAM MgFe hydrogel (E) stained with a cyclooxygenase-2 (COX-2) antibody and counterstained with hematoxylin. No significant differences were found between the injected treatments. Chondrocyte-like cell (asterisk) density is increased, and small size clones are present (arrow), indicative of early IVD degeneration. None of the cells in this NPs demonstrated positive staining for the COX-2 antibody. * Indicates significant difference at a 99% confidence level. MMP13 = matrix metallopeptidase 13, TIMP1 = tissue inhibitor of metalloproteinase 1.
Figure 6. Representative macroscopic and histopathologic image stained with alcian blue/picrosirius red of an intervertebral disc treated with NaCl (sham) (A), and glycosaminoglycan (GAG) and prostaglandin E2 (PGE₂) levels normalized for DNA in nucleus pulposus (NP) and annulus fibrosus (AF) tissue (B – C). The NP in Figure A has a bulging aspect due to the processing method. B. GAG/DNA levels were significantly higher in the NP than in the AF in all treatments. C. In all treatments PGE₂/DNA levels were significantly lower in the NP than those in the AF. Data are expressed as mean values ± standard deviation.

* Indicate significant difference at a 99% confidence level. Treatments: CXB 7.7 μM = celecoxib bolus, CR = unloaded poly-(N-isopropylacrylamide) MgFe-layered double hydroxide (LDH) hydrogel, CR + CXB 7.7 μM = pNIPAAM MgFe-LDH hydrogel loaded with 7.7 μM CXB, CR + 77 μM = pNIPAAM MgFe-LDH hydrogel loaded with 77 μM CX, CR + CXB 770 μM = pNIPAAM MgFe-LDH hydrogel loaded with 770 μM CXB.

**COX pathway and PGE₂ levels**

In all CXB-loaded hydrogels, a decrease in PGE₂/DNA levels in the NP was detected relative to the NaCl injected NPs, with a maximum reduction of 35% for the 77 μM CXB-loaded hydrogel. However, PGE₂/DNA levels in the NP as well as the AF showed high standard deviations and were not significantly different between the treatments 4 weeks post-injection. Gene expression levels of genes involved in PGE₂ biosynthesis, i.e. PTGES1, PTGES2, COX1, and COX2, showed no significant differences between treatments either. Relative gene expression levels of genes associated with inflammation, i.e. TNFA, IL1B, IL6, and IL10 were below the detection level for all conditions. Cells expressing positive immunohistochemical COX-2 staining were detected in only 3 sites out of a total of 162 (54 IVD levels). Percentages of COX-2 positive cells in the NP and VAF of two IVDs injected with 770 μM CXB-loaded hydrogel were low, 0.5% and 2% respectively, and 0.6% in an IVD injected with unloaded hydrogel. Regardless of the treatment, PGE₂/DNA levels in the NP were significantly lower than those detected in the AF ($M = -0.64$, $SD = 0.08$, CI 99% -0.85 – -0.43) (Figure 6C).
Discussion

To the authors’ knowledge this is the first study that describes biocompatibility and safe intradiscal application of a thermoreversible pNIPAAM MgFe-LDH hydrogel in vivo. The hydrogel was successfully employed as a vehicle for the delivery of a COX-2 inhibitor into the IVD. The selective COX-2 inhibitor celecoxib (CXB) was selected, as this drug is commonly used to alleviate pain symptoms associated with degenerative IVD conditions.

In vitro controlled release of CXB results into sustained suppression of PGE$_2$ levels associated with inflammation. In vitro, degradation behavior of the thermoresponsive hydrogel was comparable for different loading dosages of CXB. Although an increase in the cumulative release of CXB as percentage of the total amount loaded was detected in gels with a lower loading dose, the absolute amounts measured in the medium were comparable, demonstrating a CXB solubility-dependent release. Hydrogels with higher amounts of LDH particles showed a lower cumulative Mg release, most likely due to an increase in anionic exchange with the medium. Neither an increase in the amount of LDH particles, nor changes in the charge of Mg affected the CXB release profiles. In vitro, stimulation of 3D chondrocyte constructs with 10 ng/ml TNF-α, resulted in increased PGE$_2$ levels in the culture medium. Furthermore, in this in vitro model, we confirmed the controlled release of CXB into the medium from 0.1 mg/ml and 1 mg/ml CXB-loaded hydrogels, respectively. Unfortunately, CXB values measured by the UPLC method showed high variances most probably due to fact that the values were measured in the lower region of the UPLC detection method. TNF-α-induced PGE$_2$ production was completely inhibited by 1 mg/ml CXB-loaded pNIPAAM MgFe-LDH hydrogels, thereby proving the efficacy of the CXB controlled release system to suppress in vitro PGE$_2$ production for a prolonged period in contrast to the short-lived effect of a bolus of CXB.

Safe subcutaneous and intradiscal application of the thermoreversible pNIPAAM MgFe-LDH hydrogel. In vivo, subcutaneous injection of the hydrogel did not result in local or systemic adverse effects; histology showed a moderately irritant reaction with a shift from a pyogranulomatous reaction into a granulomatous reaction with formation of a fibrous capsule, consistent with a foreign body reaction to biomaterials. Based on these clinical and histological findings, in combination with the avascular nature of the IVD, we concluded that the hydrogel would be well tolerated when applied intradiscally. Indeed, safe intradiscal injection of thermo-responsive CXB-loaded and unloaded pNIPAAM MgFe-LDH hydrogels was demonstrated in a large animal model, i.e. chondrodystrophic dogs, with naturally occurring IVD degeneration. This was corroborated by IVD histology, further determination of the anabolic/catabolic state of the ECM and cell viability by means of gene expression and biochemical analyses. The presence of the hydrogel in the NP, in contrast to the subcutaneous location, was not accompanied by a foreign body reaction.
Histological findings in the majority of the canine IVDs were unremarkable. Notably, in one IVD injected with unloaded hydrogel, fibrous tissue in the AF was detected, whereas in another one irregular EPs and clustering of chondrocytes in the dorsal AF were present. In one of the IVDs injected with NaCl, acellular material and bulging of the ventral AF were noted. These irregularities cannot be attributed with certainty to the injections, and may also reflect spontaneous progression of IVD degeneration. This study was conducted in mildly degenerated IVDs without fissures in the AF and we demonstrated safe intradiscal injection of 30 μl hydrogel through a 29G needle. However, we did not study the ability of the pNIPAAM MgFe-LDH hydrogel to form an interface with NP tissue and therefore we cannot exclude the risk of extrusion of the biomaterial in severely degenerated IVDs that contain annular fissures.

Viability of the resident NP cells of the NP was not affected by the in vivo intradiscal application of the pNIPAAM MgFe-LDH hydrogel loaded with CXB over a wide dose range. Relative gene expression levels of the anti-apoptotic gene BCL2 were significantly downregulated in the NPs of IVDs injected with the CXB-loaded hydrogel compared with the NaCl injected IVDs. In numerous cancer cell lines overexpressing COX-2, CXB has been shown to activate the intrinsic apoptotic pathway. However, in our study, gene expression levels of other apoptotic markers, i.e. CAV1, CASP3, and FasL together with results of the TUNEL assay showed no evidence that apoptosis was affected by the intradiscal application of (un)loaded pNIPAAM MgFe-LDH hydrogel in vivo. Altogether this indicates that the differences of BCL2 on gene expression level may not be of biological relevance. In line with the aforementioned, expression levels for notochordal cell markers T, CK8 and CK18 did not differ between groups indicating that neither the injection nor the (un)loaded biomaterial had an adverse effect on IVD health.

Safe intradiscal application of the thermoreversible pNIPAAM MgFe-LDH hydrogel loaded with a wide range of CXB dosages. The pNIPAAM MgFe-LDH hydrogel loaded with CXB over a wide dose range, also appeared to be biocompatible and safe for intradiscal application at a biomolecular and biochemical level. Intradiscal injection was not associated with IVD herniation, nor with progression of degeneration. Overall, there were no significant differences between treatments on expression levels of other catabolic (MMP13) and anti-catabolic (TIMP1) genes, genes of extracellular matrix (ECM) components, i.e. ACAN, COL2A1, COL1A1, and the GAG/DNA content. Furthermore, gene expression levels of ADAMTS5 were significantly upregulated in NPs treated with the CXB bolus and the CXB-loaded hydrogel, independent of the dose, compared with NPs treated with the unloaded hydrogel, suggestive of a catabolic effect of CXB. In line with this, ADAMTS5 gene expression levels did not significantly differ between IVDs injected with unloaded hydrogels and NaCl. Our findings do not correspond with upregulated levels of
ADAMTS4 reported in bovine NPCs cultured in HA-pNIPAAM hydrogels compared with NPCs cultured in alginate beads. Nevertheless, these results should be compared to our results with care, as the relevant fold-change in gene expression was limited to a 2-fold difference in our study, and the experimental environment (i.e. in vitro versus in vivo), as well as the tissue and the composition of the hydrogel differed. Altogether, the pNIPAAM MgFe-LDH hydrogel is biocompatible and can be safely injected in the IVD without affecting the IVD health based on the overall results of histological scores, immunohistochemical indices gene expression profiles, and biochemical analyses.

Controlled release of CXB in mildly degenerated IVDs had a limited effect on PGE2 levels over the period of 28 days and there was no dose-dependent effect in a wide range of CXB concentrations. Due to technical limitations we were not able to determine the CXB tissue levels in vivo and hence cannot elaborate on the in vivo release profile of the loaded hydrogels specifically in the matrix-rich NP environment. As CXB has a relatively high protein binding capacity, the protein-rich NP might have enhanced CXB solubility, hence an increased release of CXB from the hydrogel. Given that the only available reports on the effect of CXB on PGE2 production by IVD cells are from in vitro experiments, the discussion of the results is further limited by differences between cell responses in an in vivo and an in vitro situation. Furthermore, PGE2 levels detected in these in vitro experiments are expressed in PGE2/ml medium or PGE2/total protein, and in none of them in PGE2/DNA. When correcting the PGE2 levels for protein content in the NP (Additional file 3), the results are consistent with findings in 7-day cultures of human grade 3 degenerated tissue, which showed no inhibition of PGE2 production in the presence of 1 µM CXB. Although we cannot rule out a suboptimal effect of the CXB-loaded hydrogels in the intradiscal environment, the lack of PGE2 inhibition by CXB in the current study may have been attributable to the constitutive activity of COX-1 and the absence of inflammation-induced COX-2 activity. The low percentage of COX-2 positive cells detected at the immunohistochemical level supports this theory. Furthermore, in patients with degenerative joint cartilage, treatment with CXB for a period of 28 days has been shown to have a beneficial effect on GAG turnover, mainly due to COX-2 inhibition. GAG/DNA was not significantly different for any of the treatments, which may also be associated with the absence of COX-2 activity in this model. Insufficient statistical power because of the modest sample size (N = 18) may have played a role in limiting the significance of the statistical comparisons conducted (Additional file 4). Although MR images, macroscopic, and histologic findings were consistent with mild degeneration, this phase in the degenerative cascade may actually not be associated with increased PGE2 levels, suggesting that inflammation occurs at a later time point, i.e., when disc protrusion and/or clinical signs are present. Incorporation of general COX inhibitors into the hydrogel may provide clarity on this.
Interestingly, regardless of the treatment condition, PGE$_2$/DNA levels measured in the AF in our study were significantly higher than the levels detected in the NP for all conditions, while GAG/DNA levels in the AF were significantly lower than levels in the NP, in accordance with previous studies.\textsuperscript{5, 20} In contrast, Miyamoto et al. (2006) described similar PGE$_2$/ml levels in control NP and AF cell cultures, but showed a higher production of PGE$_2$ by AF cells compared with NP cells in response to a cyclic mechanical load.\textsuperscript{49} Inflammation is thought to play an important role in the process of IVD degeneration and disease.\textsuperscript{25} Addressing the origin of inflammatory responses and hence an earlier stage of the degenerative cascade, may be more successful in suppressing inflammation and restoring IVD homeostasis. Emerging treatment strategies aiming at controlled release of anti-inflammatory medication that target, pro-inflammatory mediators, e.g. TNF-\(\alpha\) and PGE$_2$ levels in the IVD, will need to focus on IVDs in human and/or veterinary patients with clinical signs of IVD disease.\textsuperscript{16, 25, 50} PGE$_2$ levels elevated in the course of a true inflammatory response in NP and/or AF tissue may be effectively decreased by selective COX-2 inhibitors, such as CXB. However, CXB release and bioavailability in the intradiscal environment should be understood in more detail. If these issues are solved, pNIPAAM MgFe-LDH hydrogels loaded with CXB might be a promising long-term treatment in patients with herniated discs and chronic low back pain that can be minimally invasive injected into the diseased IVD assisted by fluoroscopy or computer tomography.

**Conclusion**

We have demonstrated the biocompatibility and safety of a pNIPAAM MgFe-LDH hydrogel via subcutaneous application in mice and via intradiscal administration in a large animal model. Furthermore we have shown its capability for controlled delivery of the anti-inflammatory drug CXB, resulting in suppressed PGE$_2$ levels in a TNF-\(\alpha\) stimulated 3D tissue engineered model system for up to 28 days. The controlled release of CXB from this hydrogel resulted in limited inhibition of PGE$_2$ production in a large animal model with spontaneous IVD degeneration. This may be due to the stage of degeneration rather than the efficacy of the controlled release system.

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References


Additional file 1. Synthesis and degradation of poly-(N-isopropylacrylamide) (pNIPAAM) MgFe-layered double hydroxide (LDH) hydrogels and controlled release of celecoxib (CXB) in vitro

Synthesis of poly-(N-isopropylacrylamide) (pNIPAAM) MgFe-layered double hydroxide (LDH) hydrogels
The poly(N-isopropylacrylamide) (pNIPAAM) polymer with sulfonate endgroup was synthesized through radical polymerization of NIPAAM (Sigma Aldrich, St. Louis, USA) with an initiator, 2,2'-azobis (2-methylpropionitrile) (AIBN, Fluka), and in the presence of a chain transfer reagent (CTA), HS(CH2)3SO3Na (Sigma-Aldrich), at a molar ratio NIPAAM/AIBN/CTA of 80/0.20/1. Methanol (120 ml) (ABS, Sigma-Aldrich) was added to 33.9 g of NIPAAM and 0.668 g CTA agent under nitrogen, and the mixture was stirred for 30 minutes. After addition of 123 mg of AIBN, the mixture was immediately brought to refluxing conditions. After a reaction time of 48 hours, the reaction mixture was cooled to room temperature and the solvent was removed by evaporation. The crude polymer was dissolved in 150 ml dioxane (Sigma-Aldrich) and precipitated by the drop wise addition to a fifteen-fold volume of diisopropyl ether (Sigma-Aldrich). This precipitation was repeated two times to make sure that NIPAAM monomers were removed. The precipitated polymer was filtered, washed two times with 500 ml of diethyl ether (AR, ≥99.5%), stabilized with butylhydroxytoluene (Biosolve BV, Valkenswaard, The Netherlands), and dried overnight at room temperature. This precipitation was repeated two times to make sure that NIPAAM monomers were removed (Yield 98%). The polymer was characterized by 1H NMR (1H NMR (CDCl3, 400 MHz): δ 1.0-1.2 (br, 6H), 1.3-1.9 (br, 2H), 2.1-2.2 (br, 1H), 3.9-4.1 (br, 1H), 6.0-6.6 (br, 1H)), as well as Gel Permeation Chromatography (GPC) using a GPC KD-804 column (Shodex, 300 x 8.0 mm) and polyethylene glycol calibration samples. DMF with 10 mM LiBr was used as mobile phase at a rate of 1.0 ml/min at 50°C. The GPC was equipped with a refractive index detector to determine Mn (6600 g/mol), Mw (26500 g/mol), and PDI 4.0. S-elemental analysis gave 0.65 wt% sulfur (0.70 wt% calculated).

The inorganic MgFe-LDH was synthesized by co-precipitation of the metal salts with NaOH under nitrogen atmosphere. To obtain pristine MgFe-LDH, MgCl2·6 H2O and FeCl3·6 H2O (Sigma-Aldrich) were dissolved in deionized water to a concentration of 1.05M and 0.35M, respectively, and another solution was prepared using NaOH and NaCO3 (Sigma-Aldrich) in deionized water to a concentration of 2.35M and 0.22M respectively. Both solutions were simultaneously added to a stirred beaker using syringe pumps, stirred for another 10 minutes and then dialyzed to remove excess salts (conductivity 108 μS/cm, pH 9.5). The purified MgFe-LDH suspension was aged at 110°C for 18 hours. The dispersions were then cooled and stored at room temperature. To obtain a 6 wt% LDH dispersion, dispersions were concentrated using a rotary evaporator.
Powder X-ray diffraction (pXRD) was used to study the structural properties of LDHs. PXRD spectra/patterns were obtained using an X-ray diffractometer (Philips X’Pert SR5068; PANalytical, Almelo, the Netherlands) with Cu Kα radiation (λ=1.54 Å) at 45 kV and 40 mA, and at a scanning rate of 0.8°/min. Peaks were observed at the following positions: 2θ = 11.3°(003), 22.9°(006), 34.7°(009), 37.9°, 46.8°, 59.4°(Fe), 60.8°(110). The full width at half-maximum (FWHM) of the first order peak (11.3°) was 0.36°, leading to an average size of 25 nm using the Scherrer equation. The ratio Mg:Fe was determined to be 2.6:1 using a Prodigy High Dispersion Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) system (Leeman, Illinois, USA). Standards were prepared by using multi-element (23 elements in diluted nitric acid) standard solution IV (1000 mg/l) (Merck, Darmstadt, Germany). A volume of 0.5 ml of the solutions from the degradation experiment was diluted in 100 ml aqueous 1N HNO₃, and subsequently diluted again tenfold in 1N HNO₃. In vitro release of CXB and Mg from pNIPAAM MgFe-LDH hydrogels in vitro is demonstrated in Figure 1.

**Heating rate employed in rheological analysis of the pNIPAAM MgFe-LDH hydrogels**

It is expected that after injection in the body, the hydrogel is heating up fast due to the high conductivity of the hydrogel. An estimate calculation (assuming the hydrogel has similar properties as water) gives the following: energy needed to heat up 100 μl of hydrogel = 4.185 kJ/kgK * 0.1 g * 17 K = 7.1 Joule. Heat transfer Q= k * A * dT * d = 0.6 W/mK (thermal cond. Water) * 10⁻⁴ m² (minimum surface of gel particle of 100 μl) * 5K (minimum temperature difference between LCST and body temperature)/2.88 * 10⁻³ m (max. radius of gel particle of 100 μl) = 0.11 J/s as the minimum rate of heat transfer.

To transfer 7,1 Joule max. 65 seconds are needed. Therefore, a heating rate of 15 degrees in 60 seconds appeared to be a good estimation for the real time situation. In the beginning, heating up will be faster due to a higher temperature gradient, and the fact that the hydrogel is in a needle, increasing the surface and decreasing the transfer radius. Altogether this is difficult to translate one-on-one to the rheological measurement.
Figure 1. The cumulative release (%) of celecoxib (CXB) and Mg ions from the pNIPAAM MgFe-LDH hydrogels in PBS/0.2% Tween®.

Figure 2. The cumulative release (%) of celecoxib (CXB) (A) and Mg (B) from pNIPAAM MgFe-LDH hydrogels with 1% wt CXB or 0.6% wt CXB, with single LDH or double LDH content, and with Mg_{2,5}Fe LDH, or Mg_{3}Fe LDH in PBS with 0.2% Tween 80° and once in 2%. Data are expressed as mean ± standard deviation.

Figure 3. Celecoxib (CXB) concentrations (µM) measured in medium samples of 3D chondrocyte constructs after administering a 1 µM bolus injection of CXB (CXB bolus) for 2 consecutive days and pNIPAAM MgFe-LDH hydrogels loaded with 0.1 mg/ml CXB (CR CXB). Data are expressed as mean mean ± standard deviation.
**Additional file 2. Primers used for quantitative PCR**

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Additional file 3 PGE\(_2\) normalized for total protein

Prostaglandin E\(_2\) (PGE\(_2\)) normalized for total protein (TP) in nucleus pulposus (NP) tissue. No significant differences were detected between different treatments. NaCl = sham; CXB 7.7 \(\mu\)M = celecoxib bolus; CR = unloaded poly-(N-isopropylacrylamide) pNIPAAM MgFe-layered double hydroxide (LDH) hydrogel; CR + CXB 7.7 \(\mu\)M = pNIPAAM MgFe-LDH hydrogel loaded with 7.7 \(\mu\)M CXB.
Additional file 4. Power analysis of the studies in laboratory Beagle dogs

Power analyses were performed prior to both \textit{in vivo} studies by using free software (http://www.stat.uiowa.edu/~rlenth/Power). A power analysis for the first study was based on \textit{in vivo} data from a pilot study ($N = 3$), i.e. not described in this manuscript, and for study II based on data from study I reported in this manuscript. To calculate the number of animals needed for each study, a randomized complete block design was selected via ‘balanced ANOVA’. Given that we employed this model to determine whether the controlled release of celecoxib (CXB) in an intradiscal environment would result into sustained suppression of prostaglandin E$_2$ (PGE$_2$) levels, SD[block] and SD[residual] were calculated with a linear mixed effect model on log transformed PGE$_2$/DNA nucleus pulposus (NP) data. Alpha was set at 0.05 and a Bonferroni test was selected to correct for multiple comparisons. Power was set at 0.85.

To determine the sample size for the \textit{in vivo} study I, five levels of treatment and 4 relevant comparisons between these treatments were included. SD[block] and SD[residual] were set at 0.18 and 0.35 respectively. A detectable contrast (= difference between the means log(PGE$_2$/DNA) of NaCl and controlled release (CR) + CXB 7.7 \( \mu \)M) of 0.62 was estimated and resulted in a sample size of 9 dogs. Reevaluation of the power analysis after completion of study I showed a detectable contrast of 0.29, hence a sample size that was too small. Based on the interim analysis of study I and complete reduction of PGE$_2$ \textit{in vitro} by hydrogels loaded with a 10-fold higher loading dose of CXB (77 \( \mu \)M), the study design was adapted and study II was performed. Next to hydrogels loaded with the initial concentration of CXB (7.7 \( \mu \)M), hydrogels loaded with 10- and 100-fold higher dosages of CXB were also included. To calculate the number of dogs to be included in study II, six levels of treatment and 9 relevant comparisons were included. SD[block] and SD[residual] were set at 0.28 and 0.48 respectively. A decrease of 60\% in PGE$_2$/DNA by the CXB 77 \( \mu \)M was estimated, resulting in a detectable contrast calculated at 0.91 and a sample size of 9 dogs. However, after completion of study II the calculated contrast of the second study was 0.49, indicating insufficient power to detect a difference.
Chapter 5

Inflammatory profiles in canine intervertebral disc degeneration

Nicole Willems, Anna R. Tellegen, Niklas Bergknut, Laura B. Creemers, Jeannette Wolfswinkel, Christian Freudigmann, Karin Benz, Guy C.M. Grinwis Marianna A. Tryfonidou, Björn P. Meij

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Abstract

Background
Intervertebral disc (IVD) disease is a common spinal disorder in dogs and degeneration and inflammation are significant components of the pathological cascade. Only limited studies have studied the cytokine and chemokine profiles in IVD degeneration in dogs, and mainly focused on gene expression. A better understanding is needed in order to develop biological therapies that address both pain and degeneration in IVD disease. Therefore, in this study, we determined the levels of prostaglandin E2 (PGE2), cytokines, chemokines, and matrix components in IVDs from chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs with and without clinical signs of IVD disease, and correlated these to degeneration grade (according to Pfirrmann), or herniation type (according to Hansen). In addition, we investigated cyclooxygenase 2 (COX-2) expression and signs of inflammation in histological IVD samples of CD and NCD dogs.

Results
PGE2 levels were significantly higher in the nucleus pulposus (NP) of degenerated IVDs compared with non-degenerated IVDs, and in herniated IVDs from NCD dogs compared with non-herniated IVDs of NCD dogs. COX-2 expression in the NP and annulus fibrosus (AF), and proliferation of fibroblasts and numbers of macrophages in the AF significantly increased with increased degeneration grade. GAG content did not significantly change with degeneration grade or herniation type. Cytokines interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, immune protein (IP)-10, tumor necrosis factor (TNF)-α, and granulocyte macrophage colony-stimulating factor (GM-CSF) were not detectable in the samples. Chemokine (C-C) motif ligand (CCL)2 levels in the NP from extruded samples were significantly higher compared with the AF of these samples and the NP from protrusion samples.

Conclusions
PGE2 levels and CCL2 levels in degenerated and herniated IVDs were significantly higher compared with non-degenerated and non-herniated IVDs. COX-2 expression in the NP and AF and reactive changes in the AF increased with advancing degeneration stages. Although macrophages invaded the AF as degeneration progressed, the production of inflammatory mediators seemed most pronounced in degenerated NP tissue. Future studies are needed to investigate if inhibition of PGE2 levels in degenerated IVDs provides effective analgesia and exerts a protective role in the process of IVD degeneration and the development of IVD disease.
Background

Intervertebral disc (IVD) disease is a common spinal disorder in dogs and humans and is characterized by clinical signs ranging from back pain to neurological deficits. IVD disease is preceded by IVD degeneration with a similar etiopathogenesis in dogs and humans.\(^1\) Chondrodystrophic (CD) dogs are predisposed to explosive extrusion of the nucleus pulposus (NP) (Hansen type I) of degenerated thoracolumbar and cervical IVDs, mainly between 3 and 7 years of age. Non-chondrodystrophic (NCD) dogs are predisposed to protrusion of the annulus fibrosus (AF) (Hansen type II) of degenerated lumbosacral and caudal cervical IVDs at 6 to 8 years of age, and NP extrusion of degenerated thoracolumbar IVDs.\(^2\)\(^-\)\(^5\) Hansen type II annular protrusion does occur in CD dogs, but less commonly.\(^6\)\(^,\)\(^7\)

The onset of IVD degeneration at a cellular level is characterized by a gradual replacement of notochordal cells by chondrocyte-like cells in the NP. In this respect, the NP of CD dogs contains primarily chondrocyte-like cells already by one year of age, while notochordal cells remain the predominant cell type in the NP of NCD dogs during their lifetime. In the latter, notochordal cells in some IVDs are substituted and degeneration occurs at a much later age.\(^1\)\(^,\)\(^8\)\(^,\)\(^9\) In both types of dogs, a decrease in proteoglycan content, a shift in collagen type II to collagen type I in the extracellular matrix of the NP, together with a disruption of the lamellae in the annulus fibrosus (AF) is seen during IVD degeneration.\(^10\)\(^,\)\(^11\)

Furthermore, in degenerated discs, nerve endings extend into the deeper layers of the AF and into the NP, in contrast to healthy discs, in which only the outer third of the AF is innervated. Stimulation of nociceptors in the AF and dorsal longitudinal ligament is related to pain.\(^12\)\(^,\)\(^13\) A nociceptive response can either be evoked by a mechanical or inflammatory stimulus. Various inflammatory mediators have also been suggested to play a role in the catabolic processes in human NP and AF tissue, including prostaglandin E\(_2\) (PGE\(_2\)), interleukins (IL-1\(\alpha\), IL-1\(\beta\) IL-6, IL-8), and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)).\(^14\)\(^-\)\(^16\) In NP cells from experimental CD dogs with surgically induced IVD degeneration increased levels of TNF-\(\alpha\) and IL-1\(\beta\) were shown in vitro.\(^17\) While knowledge of the involvement of inflammatory mediators in human IVD degeneration has substantially increased over the last years,\(^14\)\(^-\)\(^16\)\(^,\)\(^18\)\(^-\)\(^34\) only limited studies have focused on cytokine and chemokine profiles in IVD degeneration in dogs, and mainly focused on gene expression.\(^17\)\(^,\)\(^35\)\(^,\)\(^36\)

PGE\(_2\) is the most common prostanoid and plays an important regulatory role in physiological as well as pathological processes. It is synthesized by two cyclooxygenase (COX) isoforms, COX-1 and COX-2, by conversion of arachidonic acid into prostaglandin H\(_2\) (PGH\(_2\)) and isomerization of PGH\(_2\) to PGE\(_2\) by prostaglandin E synthases. COX-2 expression is highly restricted under physiological conditions, but can be rapidly induced in response to inflammatory stimuli and is therefore believed to play an important role in the PGE\(_2\)
production involved in degenerative processes. Current therapies of IVD disease aim at alleviating pain by administration of corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and/or opioids, by physical therapy, or by surgery. Among the numerous NSAIDs available, oral selective COX-2 inhibitors are primarily used for managing clinical signs, as they reduce inflammation and relieve pain, but cause less gastrointestinal side effects. Delivery of COX-2 inhibitors directly into the avascular IVD has been suggested as an alternative route of administration to enhance the local efficacy and to minimalize systemic side effects. A recent study in experimental CD dogs has shown the biocompatibility and safety of intradiscal injection of a hydrogel loaded with a selective COX-2 inhibitor. As PGE₂ is one of the inflammatory mediators in human IVD herniation that has been shown to sensitize nerves and induce pain, the efficacy of intradiscal delivery of NSAIDs is likely to be limited to IVD disease with a clear inflammatory profile. We hypothesize that PGE₂ levels are higher in degenerated and herniated (protruded or extruded) IVDs of CD and NCD dogs compared with non-degenerated or non-herniated IVDs. Therefore, we determined the levels of PGE₂, cytokines, chemokines, and matrix components in IVDs from CD and NCD dogs with and without clinical signs of IVD disease and correlated these to degeneration grade or herniation type. In addition, we investigated COX-2 expression in histological IVD samples of CD and NCD dogs.

Materials and methods

Collection and preparation of samples for biochemical analyses

IVDs collected post-mortem

A total of 19 IVDs, with a Thompson grade I and II, were collected from 7 laboratory (3 CD, 4 NCD) dogs that were euthanized in unrelated animal experiments (experiment numbers: DEC 2007.III.08.110, DEC 2009.III.06.050), and a total of 34 samples, with a Pfirrmann grade II, were collected from 15 laboratory (CD) dogs from previous animal experiments (DEC 2012.III.05.046, DEC 2013.III.02.017). All animal experiments were approved by the Ethics Committee of Animal Experiments (DEC) of Utrecht University. None of the dogs had a history of clinical signs of IVD disease. NP and AF tissues were isolated from the spine and collected separately, snap frozen in liquid nitrogen, and stored at -80 °C until further analysis.

IVDs collected during surgical treatment

A total of 123 IVDs were collected from 76 client-owned dogs that were referred to the University Clinic for Companion Animals in Utrecht with IVD disease that required surgical intervention. The diagnosis of IVD disease was confirmed on MRI or CT. Dogs were classified as CD and NCD and divided into subgroups, based on the Pfirrmann grade on T2-weighted MR images. In 10 samples of 6 dogs, in which no MRI was available, grading of the IVD was performed on CT images. The medical history and records of the
dogs were screened for information on prior medical treatment of IVD disease and the duration of this treatment.

**Diagnostic imaging**
Diagnostic imaging was performed in fully anesthetized client-owned dogs, according to standard practice. MRI images were obtained with a 0.2 Tesla open MRI system (Magnetom Open Viva, Siemens AG, Erlangen, Germany) by using multipurpose flex coils until 2013, and thereafter with a 1.5 Tesla scanner (Ingenia, Philips Healthcare, Best, The Netherlands) by using a small-extremity or a posterior coil. For each examination a coil was chosen that fitted around the body of the patient as closely as possible. Sagittal T2-weighted (T2W) images were acquired using a turbo-spin echo pulse sequence with the following parameters: repetition time = 2500 - 3048 ms, echo time = 110 - 120 ms, field of view = 50 x 160/160 x 350 mm, acquisition matrix = 100 x 256/200 x 235 mm, voxel size = 0.6 x 0.8/0.8 x 1.03 mm slice thickness = 2 – 2.5 mm. CT images were obtained with a third-generation single-slice helical CT-scanner (Philips Secura). Contiguous 2 mm thick slices with 1 mm overlap were obtained with exposure settings of 120 kV and 260 mA.

**Surgical treatment**
Client-owned dogs were anesthetized according to standard of care. Collection of IVDs was achieved through standard surgical procedures, depending on the location of disc herniation: ventral decompression in the cervical area, dorsolateral hemilaminectomy in the thoracolumbar area, and dorsal laminectomy in the lumbosacral area. In dogs with nuclear extrusion (Hansen type I), free NP material was collected from the epidural space in the spinal canal, and AF material was collected during ventral fenestration preceding the ventral decompression for cervical disc herniations, or when an additional lateral fenestration was performed after hemilaminectomy for thoracolumbar disc herniations. In dogs with lumbosacral annular protrusion (Hansen type II), partial discectomy consisting of annulotomy and nucleotomy, allowed separate collection of NP and/or AF tissue. In 3 dogs, an adjacent IVD was fenestrated, and AF and/or NP material was collected. The treatment decision (discectomy, nucleotomy, fenestration) was taken during surgery and depended on the state of the AF and the position of the NP. Each surgeon documented the type of herniation (NP extrusion (Hansen type I) or AF protrusion (Hansen type II)) and type of collected material (NP or AF) in the surgical report.

NP and/or AF tissues were collected in separate vials during surgery, snap frozen into liquid nitrogen within minutes after collection, and subsequently stored at -80 °C until further analysis. Details of the samples are shown in Table 1 and in Additional file 1.
Table 1. Sample classification details.

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^a samples collected from experimental dogs
^b 9 samples collected from experimental dogs (non-surgical); 17 samples collected in a previous study
^c 4 samples collected from experimental dogs (non-surgical); 17 samples collected in a previous study
^d 1 sample collected via fenestration
^e samples collected from experimental dogs; 17 samples collected in a previous study

CD = chondrodystrophic, NCD = non-chondrodystrophic, mths = months, yrs = years, NA = not available
Biochemical analyses of IVDs collected post-mortem and intra-operatively

Prior to analyses, samples were weighed, and 400 μl and 750 μl lysis buffer (cOmplete lysis M EDTA buffer, Roche diagnostics Nederland B.V., Almere, The Netherlands) was added to NP and AF tissue, respectively. Tissues were lysed in a TissueLyser II (Qiagen, Venlo, The Netherlands) for 2x 60 s at 20 kHz. After centrifugation for 15 minutes at 14.000 g, the volume of the supernatant of each sample was measured and separated from its pellet. A volume of 80 μl was filtered over a 0.22 μm nylon spin-X centrifuge tube filter (8169, Costar, Corning Incorporated, NY, USA) and stored at -80°C in aliquots for cytokine measurements.

Glycosaminoglycan and DNA assays

To determine GAG and DNA, supernatants and pellets were digested in a papain buffer (250 μg/ml papain (P3125-100 mg, Sigma-Aldrich) + 1.57 mg cysteine HCL (C7880, Sigma-Aldrich)) at 60 °C overnight. The 1.9-dimethylmethylene blue (DMMB) assay was used to determine GAG content. A volume of 16 mg DMMB (341088 Sigma-Aldrich) was added to 5 ml 100% ethanol and incubated overnight on a roller bench. A solution of 2.37 g NaCl and 3.04 g glycine in 1 l distilled water with a pH set at 3.00 was sterilized by using a 0.22 μm syringe filter (SLGSV255F Millex-GS Syringe Filter Unit, Merck Millipore, Darmstadt, Germany), added to the DMMB solution, and stored at 4 °C, protected from light. Pellets were diluted 1:1000 and supernatants 1:150 in PBS-EDTA. A volume of 100 μl of the dilutions and standards was pipetted into a 96-wells microplate (655199 PS microplate, Greiner Bio-One, Alphen aan den Rijn, Netherlands), and prior to spectrophotometric analysis, 200 μl of DMMB was added to each well. The ratio of absorption at 540 to 595 nm was measured by using a microplate reader (Multimode detector DTX 880, Beckman Coulter). Chondroitin sulphate from shark cartilage (C4384, Sigma-Aldrich) was used as a standard to calculate GAG concentrations. The Quant-iTTM dsDNA Broad-Range assay kit in combination with a Qubit Fluorometer (Invitrogen, Carlsbad, USA) was used according to the manufacturer’s protocol to determine the DNA content.

PGE2 and cytokine assays

PGE2 levels were determined in the supernatants by using a colorimetric competitive enzyme immunoassay kit (PGE2 high sensitivity EIA kit, ENZO Life Sciences BVBA, Antwerp, Belgium). A magnetic canine cytokine bead panel based on Luminex® xMAP® technology (#CCYTOMAG-90K/CCYTOMAG-90K-PX13); Milliplex® MAP kit, Millipore Corporation, Billerica, USA) was used to measure twelve different cytokines and chemokines in supernatants: TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 10 (CXCL10). Supernatants were
diluted 1:2 and were measured according to the manufacturer’s instructions. All biochemical values were corrected for weight of the sample.

**Collection of post-mortem IVDs for histology**

**IVDs collected post-mortem**

Post-mortem, 37 IVDs were collected from vertebral columns of 16 client-owned dogs that were euthanized for diseases other than IVD disease and submitted for necropsy to the Department of Pathobiology at the Faculty of Veterinary Medicine, Utrecht University, and from 9 experimental dogs in unrelated cardiovascular experiments (DEC 2007.II.01.029, DEC 2011.07.065). Permission to collect material from the client-owned dogs was granted by the owners. None of the dogs had a reported history of back problems. Details of the dogs of which material was collected for histology are shown in Table 1.

**Diagnostic imaging**

Within 24 hours after euthanasia or death, the vertebral column (T11 – S1) was harvested by using an electric multipurpose saw (Bosch, Stuttgart, Germany). Within 1 hour after dissection, sagittal T2W MR images were obtained with a 0.2 Tesla open MRI system (Magnetom Open Viva, Siemens AG) as described earlier. All lumbar IVDs were graded on midsagittal T2W images according to the Pfirrmann score by two independent investigators (NW, AT). 42

**Histology and immunohistochemistry**

After scanning, all muscles were removed and the vertebrae were transected transversely with a band saw (EXAKT tape saw, EXAKT Advanced Technologies GmbH, Norderstedt, Germany), resulting in spinal units (endplate – IVD – endplate). These units were then transected sagittally into two halves by using a diamond band pathology saw (EXAKT 312 saw; EXAKT diamond cutting band 0.1 mm D64; EXAKT Advanced Technologies GmbH, Norderstedt, Germany). Midsagittal slices (3 – 4 mm) were cut from one half and fixed in 4% neutral buffered formaldehyde and decalcified in EDTA. Samples were dehydrated in graded alcohol series, rinsed in xylene, and embedded in paraffin. Sections (5 μm) were cut, deparaffinized and rehydrated, and stained with both hematoxylin (109249, Merck)/eosin (115935, Merck), and with picrosirius red (saturated aqueous picric acid: 36011, Sigma-Aldrich, sirius red: 8015, Klinipath)/alcian blue (alcian blue: 05500, Sigma-Aldrich; glacial acetic acid: 100063, Merck). Histological sections were assessed for the presence of inflammatory cells, and evaluated according to a histological grading scheme described by Bergknut et al. 45

Immunohistochemistry for COX-2 was performed on 5 μm sections mounted on KP plus glass slides (Klinipath B.V., Duiven, The Netherlands). After deparaffinization and
Inflammatory profiles in canine intervertebral disc degeneration

Rehydration sections were treated with Dual Endogenous Enzyme Block (S2003, Dako, California, USA) for 10 min at room temperature to block nonspecific endogenous peroxidase, followed by 2 washing steps of each 5 min with tris buffered saline containing 1% Tween 20® (TBS-T). Sections were treated with TBS bovine serum albumin (BSA) 5% solution to block non-specific binding for 60 minutes at room temperature. Subsequently they were incubated with a primary mouse anti-human monoclonal COX-2 antibody (#160112 Clone CX229, Cayman, Ann Arbor, USA) diluted 1:800 in TBS-BSA 5% overnight at 4°C. The following day sections were incubated with peroxidase-labelled polymer (K4007; Envision anti-mouse, Dako) and antibody binding was visualized by using diaminobenzidine (DAB; K4007; Dako). Sections were counterstained with hematoxylin solution (Hematoxylin QS, Vector, Peterborough, UK), rehydrated and mounted in permanent mounting medium. The percentage of COX-2 positive chondrocytes in the NP, and in the dorsal AF (DAF) was determined by manual counting by a blinded independent investigator (AT).

Statistical analyses

Data were analyzed by using R statistical software, package 2.15.2. A multiple linear regression model was used to analyze the effect of multiple explanatory variables on corrected PGE₂, GAG, and DNA levels for the wet weight of the tissues. Furthermore, in order to be able to compare this study with previous reports, PGE₂ levels were also corrected for DNA content. Data were logarithmically transformed to achieve normality. Two separate models were employed to investigate the association of explanatory variables ‘grade’ (Pfirrmann grade I – IV) and ‘herniation’ (NP in situ, NP extrusion, AF protrusion) with inflammatory parameters. Variables incorporated into both models were ‘dog’ (CD, NCD), ‘tissue’ (NP and AF), ‘treatment’ (no treatment, NSAID administered less than (<) 1 wk, NSAID administered more than (>) 1 wk, corticosteroids (cort) < 1 wk, cort > 1 wk, other) and their interaction. Residual plots and quantile-quantile (QQ)-plots were used to check the critical assumptions of linearity, equal variance at all fitted values and the assumption of normally distributed residuals. The Cox proportional hazards regression model was used for analysis of the COX-2 values, that did not approximate a normal distribution after log transformation. ‘Grade’ (Pfirrmann grade I – IV) and ‘breed’ (CD, NCD) and their interaction were incorporated into this model. Calculations were performed on values distracted from 100%. In the absence of COX-2 positive cells the sample was set at 100% and right censored. Histological reactive changes in the IVDs were statistically evaluated by using the nonparametric Kruskal-Wallis test, followed by a Mann-Whitney U-test. The Spearman’s correlation coefficient was calculated to estimate the correlation between the presence of inflammatory cells (‘yes’ or ‘no’) and COX-2 positive cells.
For all statistical models, regression coefficients were estimated by the maximum likelihood method. Model selection was based on the lowest Akaike Information Criterion (AIC). Confidence intervals were calculated and stated at the 99% confidence level to correct for multiple comparisons. Differences between treatments were considered significant if the confidence interval did not include 0, whereas hazard ratios were considered significant if the confidence interval did not include 1. Significant differences and the corresponding confidence intervals are represented in Additional file 2.

Results

Extracellular matrix components and inflammatory profiles in relation to stage of degeneration

GAG content normalized for wet weight did not significantly change with degeneration grade according to Pfirrmann (Figure 1A and 1B). In grade IV + V samples the GAG content in the NP was significantly lower than in the AF (Figure 1A). DNA expressed as μg/mg wet weight was significantly lower in grade II samples compared with grade IV + V samples (Figure 1B). Due to sample limitations, samples that were above the upper range of the PGE₂ assay (> 1000 pg/ml) were set at 1000 pg/ml. PGE₂ levels normalized for wet weight were significantly lower in grade I NP samples compared with those in grade II, III, and IV + V NP samples (Figure 2A). PGE₂ levels normalized for DNA were significantly lower in grade I samples compared with grade II, III, and IV + V samples regardless of the tissue origin (NP/AF), dog type (CD/NCD), or treatment group (no treatment, NSAID < 1 wk, NSAID > 1 wk, cort < 1 wk, cort > 1 wk, other) (Figure 2B). Cytokines IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10, TNF-α, and GM-CSF were not detectable in the samples. Chemokine CCL2 was measured in 66/120 samples and chemokine CXCL1 in 119/120 samples. CXCL1 and CCL2 expressed as pg/gram wet weight did not significantly change with degeneration (Figure 2C and 2D). There were no significant differences between treatment groups.

Extracellular matrix components and inflammatory profiles in relation to herniation of NP and AF

GAG and DNA (Figure 1C and 1D) and CXCL1 (Figure 2D and 2E) expressed as pg/gram wet weight were not significantly different between herniation groups. PGE₂ levels normalized for either DNA content or wet weight, were significantly lower in non-herniated samples compared with extruded and protruded samples of NCD dogs, regardless of the tissue origin (NP/AF), or treatment group (no treatment, NSAID < 1 wk, NSAID > 1 wk, cort < 1 wk, cort > 1 wk, other). CCL2 levels in the NP from extruded samples were significantly higher compared with the AF of these samples and the NP from protrusion samples regardless of the dog breed (CD/NCD) (Figure 2F). There were no significant differences between biochemical parameters of CD and NCD dogs or treatment groups.
Figure 1. Mean ± standard deviation glycosaminoglycan (GAG) and DNA content normalized for weight in the nucleus pulposus (NP) and annulus fibrosus (AF) per Pfirrmann grade (A and B) and per herniation (C and D). A. GAG/weight levels were significantly higher in the AF compared with the NP in grade IV + V samples. B. DNA/weight was significantly lower in the NP of grade II samples compared with grade IV + V. C and D. Normalized GAG and DNA levels did not significantly differ between herniation groups. No significant differences were shown between dog (chondrodystrophic, non-chondrodystrophic) or treatment (no treatment, NSAID < 1 wk, NSAID > 1 wk, corticosteroids (cort) < 1 wk, cort > 1 wk, other) groups, hence these groups are not shown separately. ** Indicates significant difference at a 99% confidence level.

Pfirrmann grade II samples from this study were compared with Pfirrmann grade II samples obtained from experimental CD dogs (Figure 3A). As sample weights were not available in the previous study, PGE$_2$ was normalized for DNA. In this combined Pfirrmann grade II dataset, PGE$_2$/DNA in the NP was significantly higher in extruded samples compared with Pfirrmann grade II IVDs with the NP in situ. To compare this combined Pfirrmann grade II dataset to the complete dataset, we have normalized PGE$_2$ for DNA (Figure 3b and 3c).
Figure 2. Mean + standard deviation prostaglandin E$_2$ (PGE$_2$) and chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1) levels normalized for weight in the nucleus pulposus (NP) and annulus fibrosus (AF) per Pfirrmann grade (A, B, C) and per herniation (D, E, F). A. PGE$_2$ levels expressed as pg/mg wet weight in grade I NP samples were significantly lower compared with grade II, III, and IV + V NP samples. B and C. CCL2 and CXCL1 levels normalized for weight did not significantly change with degeneration. D. PGE$_2$ levels did not significantly differ in the NP and AF between the three herniation groups. E. CXCL1 levels did not significantly differ between herniation groups. F. CCL2 levels normalized for weight in the NP from extruded samples were significantly higher compared with the AF of these samples and the NP from protruded samples. No significant differences were shown between treatment (no treatment, NSAID < 1 wk, NSAID > 1 wk, corticosteroids (cort) < 1 wk, cort > 1 wk, other) groups, hence these groups are not shown separately. ** Indicates significant difference at a 99% confidence level.
Figure 3. Mean + standard deviation PGE$_2$ levels normalized for DNA content in the nucleus pulposus (NP) and annulus fibrosus (AF) in Pfirrmann grade II samples obtained from experimental chondrodystrophic (CD) dogs (A), and in the complete dataset (CD and nonchondrodystrophic (NCD) dogs) per Pfirrmann grade (B), and per herniation (C). A. PGE$_2$ levels expressed as pg/μg DNA in the NP of CD dogs were significantly higher in protruded grade II samples compared with NP in situ samples. B. PGE$_2$ levels normalized for DNA were significantly lower in grade I samples compared with grade II, III, and IV + V samples regardless of the tissue origin (NP/AF), dog group (CD/NCD), or treatment group (no treatment, NSAID < 1 wk, NSAID > 1 wk, corticosteroids (cort) < 1 wk, cort > 1 wk, other). C. PGE$_2$ levels expressed as pg/μg DNA were significantly lower in non-herniated samples compared with extruded and protruded samples in NCD dogs, regardless of the tissue origin (NP/AF), or treatment group (no treatment, NSAID < 1 wk, NSAID > 1 wk, corticosteroids (cort) < 1 wk, cort > 1 wk, other). ** indicates significant difference at a 99% confidence level.

Histology and COX-2 expression

Histological scores according to the grading scheme by Bergknut et al. ranged from 7 – 12 (median = 8) for Pfirrmann grade I, 8 – 27 (median = 14) for Pfirrmann grade II, 14 – 20 (median =19) for Pfirrmann grade III, and 18 – 26 (median = 21) for Pfirrmann grade IV + V. In 5/37 IVDs from 3/16 dogs ventral bone formation was seen in grade I – V IVDs. Histology revealed no inflammatory cells or fibroblasts in the NP of Pfirrmann grade I – V IVDs, and in the dorsal AF of Pfirrmann grade I IVDs. However, in higher degeneration grades, focal infiltration of macrophages, proliferation of fibroblasts and capillaries were detected in the dorsal and/or the ventral ligament, extending into the outer layers of the dorsal and ventral AF, respectively (Figure 4). Macrophages and proliferation of fibroblasts were present in 0% (0/10), 10% (1/10), 83% (5/6) and 55% (6/11) of the IVDs scored a Pfirrmann grade I, II, III, IV + V, respectively. Numbers of macrophages and fibroblasts in grade IV + V IVDs were significantly higher than in grade I, and in grade III significantly higher than in grade I and II. Protrusion of the AF was seen in a grade II and a grade IV + V IVD.
Figure 4. Representative histological images of the annulus fibrosus (AF) of intervertebral discs (IVDs) graded according to Pfirrmann stained with a cyclooxygenase-2 (COX-2) antibody and counterstained with hematoxylin. A. The dorsal AF of a non-degenerated Pfirrmann grade 1 IVD consisted of well-organized lamellae with COX-2 negative spindle-shaped fibroblasts (asterisks). B. In the dorsal AF of a degenerated Pfirrmann grade IV IVD lamellar organization was lost and COX-2 negative chondrocytes (arrowheads) as well as COX-2 positive chondrocytes (arrows) were present. C. The dorsal AF of a Pfirrmann grade V IVD consisted of COX-2 negative chondrocytes (arrowheads), whereas COX-2 positive macrophages (open arrows) were situated in the dorsal ligament. Percentages of COX-2-positive cells in the NP and dorsal AF of grade I and grade II tissue were significantly lower compared with the NP and dorsal AF of grade IV + V samples (Figure 5). The presence of macrophages and fibroblasts in the dorsal AF was moderately correlated (Spearman’s ρ = 0.4, p-value = 0.003) with COX-2 positive cells in the dorsal AF.

Figure 5. Percentage of COX-2-positive cells in the nucleus pulposus (NP) and annulus fibrosus (AF) per Pfirrmann grade. The NP and AF of grade I and grade II samples were significantly lower compared with the NP and AF grade IV + V samples. ** Indicates significant difference at a 99% confidence level

Discussion
To our knowledge this is the first study that describes levels of COX-2, PGE₂, cytokines, chemokines, and matrix components in IVDs from CD and NCD dogs with and without clinical signs of IVD disease and degeneration. PGE₂ levels were significantly higher in degenerated IVDs compared with non-degenerated IVDs, and they were also higher in herniated (protruded and extruded) IVDs from NCD dogs compared with non-herniated IVDs of NCD dogs. In contrast to PGE₂ levels in Pfirrmann grade II IVDs from CD dogs with (a limited number of) protruded IVDs, PGE₂ levels in extruded IVDs were not significantly different from IVDs with the NP in situ. Furthermore, COX-2 protein expression was significantly higher in degenerated IVDs compared with non-degenerated IVDs. These results are consistent with findings in herniated human lumbar IVD cells that produced increased PGE₂ levels spontaneously in vitro compared with PGE₂ levels in control IVD cells.¹⁵
Contrary to PGE2 levels, COX-2 expression in the NP and AF and numbers of macrophages in the dorsal and ventral ligaments were increased in advanced stages of degeneration. Histological results of non-herniated degenerated IVDs in our study are consistent with histological findings described in studies on canine herniated IVDs. In extruded IVDs an acute inflammatory reaction has been described, characterized by neutrophils and macrophages, while in protruded IVDs a more chronic inflammatory reaction has been described, characterized by macrophages, lymphocytes and plasma cells.47, 48

Macrophages do not only have a phagocytic function, but secret next to cytokines also a number of growth factors, e.g. fibroblast growth factor, transforming growth factor beta, that can induce neovascularization and mediate cell proliferation and differentiation.49 The focal infiltrates of macrophages, proliferation of fibroblasts and capillaries, and the new bone formation as was seen histologically in some IVDs are reactive tissue changes that might reflect a process of tissue repair. Although a physiological inflammatory response to aseptic tissue injury primarily serves to promote tissue repair, macroscopic findings may reflect an excessive inflammatory response. This response may have detrimental effects on tissue integrity, and may contribute to the pathogenesis of IVD degeneration and/or disease.

Significantly higher PGE2 levels in degenerated NP tissues compared with non-degenerated IVDs were observed. Although not significantly different, PGE2 levels and COX-2 expression in grade II and IV + V IVDs were consistently higher in the NP of degenerated IVDs compared with AF, while the contrary was true for non-degenerated IVDs. In Pfirrmann grade II samples from CD dogs, PGE2 levels in the NP were significantly higher in Pfirrmann grade II IVDs with protrusion compared with IVDs with the NP in situ. This may indicate that the production of inflammatory mediators is more pronounced at the NP level. We cannot exclude that NP and AF cells respond differently to inflammatory stimuli and mechanic stress and hence produce different levels of PGE2, as also suggested by others based on in vitro experiments in rat IVD cells.50 Cytokine and chemokine profiles in this study are largely consistent with limited veterinary publications. The significantly increased CCL2 levels in NP tissue of dogs with Hansen type I herniation compared with AF tissue and NP tissue in dogs with Hansen type II herniation are in line with other studies reporting upregulated gene expression levels of CCL2 in dogs with extrusion of the NP.35 Furthermore, increased CCL2 protein expression and CCL2 production levels have been reported in human prolapsed IVDs.28 No studies in canine tissues, and only limited studies in human tissues, have determined cytokine and/or chemokine levels by using a (multiplex) sandwich immunoassay, and have shown increased levels of IFN-γ, IL-1, TNF-α, and CCL2, in epidural lavage fluid and in cell culture media, which complicates comparison of results.51-53 Cytokine levels of IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10, TNF-α, and GM-CSF were not detected in NP and AF tissues of this study, consistent with
downregulated gene expression levels of IL-2, IL-6, IL-10, and TNF-α in herniated canine IVDs. Nevertheless, our findings seem to be in contrast with increased protein and gene expression levels of TNF-α and IL-1 in human IVDs. Given the short half-life of TNF-α and cytokines, and that all samples collected from degenerated IVDs were obtained during surgery and in several cases after flushing of the spinal canal, we cannot rule out degradation of cytokines/chemokines by the collection, preparation, and storage process. Although several studies have shown that IVD cells have the capacity to produce PGE₂, we cannot rule out that PGE₂ levels were influenced by infiltration from the epidural space.

Despite elevated PGE₂ levels in degenerated NP tissue in this study, GAG content was not significantly different between healthy and degenerated IVDs, while severely degenerated AFs (grade IV + V) had a higher GAG content compared with the NP. The latter may be explained by the presence of GAG-producing chondrocytes in the AF, known to be present in later stages of degeneration, or by the presence of unidentified GAG-rich herniated NP and/or inner AF material in AF samples. These findings are in contrast with the decrease in GAG content with increasing IVD degeneration described in literature. One plausible explanation for this discrepancy lies in the scoring system of degeneration prior to surgery and the matrix heterogeneity of the degenerated NP tissue, discussed in detail below. Interestingly, cell density (DNA/weight) in our study was significantly higher in the NP of severely degenerated IVDs compared with mildly degenerated IVDs. These findings touch upon findings in human IVD degeneration, in which cell density in the inner AF and NP of severely degenerated (Thompson grade V) specimens was significantly higher compared with lower grades.

The results on the effects of PGE₂ on proteoglycan metabolism are conflicting. PGE₂ at concentrations much lower than those involved in inflammation have been demonstrated to be chondroprotective. PGE₂ has been described to have anti-catabolic effects by downregulating the expression and synthesis of IL-1, TNF-α, and matrix metalloproteinases (MMPs), and to have anabolic effects by to inducing the expression, synthesis and secretion of IGF-I, and stimulating collagen and proteoglycan synthesis, important factors in anabolic processes. In vitro, low concentrations of PGE₂ have been described to stimulate proteoglycan synthesis in rat chondrocytes, whereas higher doses have been described to decrease proteoglycan synthesis in NP cells. Furthermore, degradation of proteoglycans was not inhibited by a range of PGE₂ concentrations in osteoarthritic chondrocytes. These possible protective effects of PGE₂ might have resulted in preservation of GAG content in the course of IVD degeneration. Nevertheless, these results should be interpreted with care, as GAG content of the studied tissues may have been affected by confounding factors explained below.
There are several confounding factors that may affect the results in the current study, including the factors that influence the scoring system of degeneration and the matrix heterogeneity of the degenerated NP tissue. Extruded NP tissue displaced into the vertebral canal results in narrowing of the disc space and a T2-hypointense area within the IVD on MRI. Hence, we cannot exclude that prior to the extrusion incident the IVD may have been assessed with a lower Pfirrmann score. Moreover, in CD dogs, calcification of the NP could have negatively influenced the signal intensity in the NP. In addition, in both CD and NCD dogs, IVDs may have been graded falsely higher due to hemorrhage or inflammation, that may have influenced the appearance of the IVD on MR images. Matrix heterogeneity is common in degenerating NP tissue. In human IVDs several disc-specific locations are described with a high variation in GAG and water content, suggestive of focal damage and degeneration. Although this has not yet been described in dogs, we cannot rule out that tissues collected during surgery may have originated from specific GAG-rich areas in the IVD, that inherently are more prone to extrusion/protrusion compared with degenerated fibrotic tissue. Furthermore, due to sample limitations PGE2 values higher than 1000 pg/ml could not be measured reliably, but could have resulted in an underestimation of the highest samples. Lastly, a relatively high percentage of dogs in this study was treated prior to surgery with anti-inflammatory drugs, e.g. NSAIDs and corticosteroids. Dogs that did not respond to anti-inflammatory drugs initially, were treated with other drugs, e.g. opioids, GABA-agonists. Although treatment groups were categorized, duration of treatment and dosages used showed a high variation, and might have had an influence on the results.

From a clinical perspective, decompression surgery is recommended if dogs present with clinical signs, and diagnostic work-up indicates compression of neural tissue (spinal cord and/or nerve roots) due to extruded material. With regard to an intradiscal application that provides controlled release of an anti-inflammatory drug, future studies should focus on protruded IVDs. Obviously, this would indicate development of an application in NCD dogs, as disc protrusion rarely occurs in CD dogs. IVDs ideally should be early degenerated (Pfirrmann grade II – III), without irreversible anatomical malformations due to degenerative changes.

**Conclusion**

In this study we have shown that PGE2 levels, and CCL2 levels in degenerated and herniated tissues were significantly higher compared with non-degenerated and non-herniated tissues. COX-2 expression in the NP and AF and numbers of macrophages in the AF increased with advancing degeneration stages. Although macrophages invade the dorsal and ventral AF as degeneration progresses, the production of inflammatory mediators seems most pronounced in degenerated NP tissue. Future studies are needed
to investigate if inhibition of PGE$_2$ levels in degenerated IVDs provide effective analgesia and exerts a protective role in the process of IVD degeneration and the development of IVD disease.

**Acknowledgements**

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References


**Additional file 1.** A more detailed representation of included samples in addition to Table 1 in the original article

NP = nucleus pulposus, CD = chondrodystrophic, Extr = nuclear extrusion, Protr = annular protrusion, NCD = non-chondrodystrophic, AF = annulus fibrosus, NSAID = non-steroidal anti-inflammatory drug, Cort = corticosteroids, NA = not available

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<th>AF CD</th>
<th>NP in situ/Extr/Protr</th>
<th>Treatment</th>
<th>AF NCD</th>
<th>NP in situ/Extr/Protr</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV+V</td>
<td>13</td>
<td>1</td>
<td>1: NSAID&lt;1 wk</td>
<td>10</td>
<td>0</td>
<td>3: NSAID&lt;1 wk</td>
<td>9</td>
<td>1</td>
<td>1: Cort&lt;1 wk</td>
<td>14</td>
<td>0</td>
<td>1: NSAID&lt;1 wk</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2: NSAID&lt;1 wk</td>
<td></td>
<td>7</td>
<td>3</td>
<td>2: NSAID&lt;1 wk</td>
<td>8</td>
<td>2</td>
<td>2: No</td>
<td>4</td>
<td>0</td>
<td>1: NSAID&lt;1 wk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4: NSAID&gt;1 wk</td>
<td></td>
<td>3</td>
<td>3: NSAID&gt;1 wk</td>
<td></td>
<td></td>
<td>1: Cort&gt;1 wk</td>
<td>2: NSAID&lt;1 wk</td>
<td>4</td>
<td>3</td>
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<tr>
<td></td>
<td></td>
<td>2: Cort&lt;1 wk</td>
<td></td>
<td>0</td>
<td>2: Other</td>
<td></td>
<td></td>
<td>1: Other</td>
<td>1: Cort&gt;1 wk</td>
<td>7</td>
<td>2</td>
<td>1: Cort&gt;1 wk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1: Cort&gt;1 wk</td>
<td></td>
<td></td>
<td>2: NA</td>
<td></td>
<td></td>
<td>1: Other</td>
<td>1: Other</td>
<td>12</td>
<td>4</td>
<td>1: Other</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2: Other</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>10</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>26</td>
<td></td>
<td>36</td>
<td>9</td>
<td></td>
<td>47</td>
<td>24</td>
<td></td>
<td>41</td>
<td>10</td>
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<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NP = nucleus pulposus, CD = chondrodystrophic, Extr = extrusion, Protr = protrusion, NCD = non-chondrodystrophic, AF = annulus fibrosus, NSAID = non-steroidal anti-inflammatory drug, Cort = corticosteroids, NA = not available.
Additional file 2. Significant differences and confidence intervals of statistical analyses

Tables 1, 2, 3, and 4 represent significant differences and confidence intervals of statistical analyses. Figure numbers in the tables correspond to figures shown in the main article.

**Table 1.** Significant differences and confidence intervals of statistical analyses of glycosaminoglycan (GAG) and DNA content normalized for weight in the nucleus pulposus (NP) and annulus fibrosus (AF) per degeneration grade and per herniation type, corresponding to Figure 1 in the main article.

<table>
<thead>
<tr>
<th>Condition vs Condition</th>
<th>Estimated coefficient</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degeneration 1A. GAG/weight</td>
<td>NP grade IV+V vs AF grade IV+V</td>
<td>0.65</td>
<td>0.05 – 1.25</td>
</tr>
<tr>
<td>Degeneration 1B. DNA/weight</td>
<td>NP grade II vs NP grade IV+V</td>
<td>1.13</td>
<td>0.22 – 2.04</td>
</tr>
</tbody>
</table>

**Table 2.** Significant differences and confidence intervals of statistical analyses of prostaglandin E₂ (PGE₂) and chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1) levels normalized for weight in the nucleus pulposus (NP) and annulus fibrosus (AF) per Pfirrmann grade and per herniation type, corresponding to Figure 2 in the main article.

<table>
<thead>
<tr>
<th>Condition vs Condition</th>
<th>Estimated coefficient</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degeneration 2A. PGE₂/weight</td>
<td>NP grade II vs NP grade I</td>
<td>2.54</td>
<td>1.10 – 3.99</td>
</tr>
<tr>
<td></td>
<td>NP grade III vs NP grade I</td>
<td>2.84</td>
<td>1.28 – 4.41</td>
</tr>
<tr>
<td></td>
<td>NP grade IV vs NP grade I</td>
<td>2.67</td>
<td>1.20 – 4.14</td>
</tr>
<tr>
<td>Herniation 2D. PGE₂/weight</td>
<td>Extrusion NCD vs In situ NCD</td>
<td>1.85</td>
<td>0.31 – 3.40</td>
</tr>
<tr>
<td></td>
<td>Protrusion NCD vs In situ NCD</td>
<td>1.98</td>
<td>0.79 – 3.17</td>
</tr>
<tr>
<td>2F. CCL2/weight</td>
<td>NP extrusion vs AF extrusion</td>
<td>2.07</td>
<td>0.64 – 3.51</td>
</tr>
<tr>
<td></td>
<td>NP extrusion vs NP protrusion</td>
<td>1.33</td>
<td>0.21 – 2.45</td>
</tr>
</tbody>
</table>

*NCD = non-chondrodystrophic*
Table 3. Significant differences and confidence intervals of statistical analyses of prostaglandin E₂ (PGE₂) normalized for DNA content in the nucleus pulposus (NP) and annulus fibrosus (AF) in Pfirrmann grade II samples obtained from experimental chondrodystrophic (CD) dogs, and in the complete dataset (CD and nonchondrodystrophic (NCD) dogs) per Pfirrmann grade, and per herniation type, corresponding to Figure 3 in the main article.

<table>
<thead>
<tr>
<th>Condition vs Condition</th>
<th>Estimated coefficient</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3A. PGE₂/DNA grade II CD dogs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP protrusion NP in situ</td>
<td>2.08</td>
<td>1.06 – 3.10</td>
<td>99</td>
</tr>
<tr>
<td><strong>Degeneration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade II Grade I</td>
<td>2.17</td>
<td>1.18 – 3.17</td>
<td>99</td>
</tr>
<tr>
<td>Grade III Grade I</td>
<td>2.13</td>
<td>1.04 – 3.21</td>
<td>99</td>
</tr>
<tr>
<td>Grade IV Grade I</td>
<td>1.47</td>
<td>0.46 – 2.48</td>
<td>99</td>
</tr>
<tr>
<td><strong>Herniation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extrusion NCD In situ NCD</td>
<td>1.85</td>
<td>0.31 – 3.40</td>
<td>99</td>
</tr>
<tr>
<td>Protrusion NCD In situ NCD</td>
<td>1.98</td>
<td>0.79 – 3.17</td>
<td>99</td>
</tr>
</tbody>
</table>

Table 4. Significant differences and confidence intervals of statistical analyses performed on cyclooxygenase-2 (COX-2) expression data in the nucleus pulposus (NP) and annulus fibrosus (AF) per Pfirrmann grade, corresponding to Figure 5 in the main article.

<table>
<thead>
<tr>
<th>Condition vs Condition</th>
<th>Estimated hazard ratio (HR)</th>
<th>Confidence interval (CI)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP grade IV + V NP grade I</td>
<td>34.3</td>
<td>2.09 – 562.54</td>
<td>99</td>
</tr>
<tr>
<td>NP grade IV + V NP grade II</td>
<td>7.40</td>
<td>1.39 – 39.46</td>
<td>99</td>
</tr>
<tr>
<td>AF grade IV + V AF grade I</td>
<td>27.32</td>
<td>1.78 – 419.75</td>
<td>99</td>
</tr>
<tr>
<td>AF grade IV + V AF grade I</td>
<td>4.92</td>
<td>1.64 – 14.79</td>
<td>99</td>
</tr>
</tbody>
</table>
Pedicle screw-rod fixation: a feasible treatment for dogs with severe degenerative lumbosacral stenosis

Anna R. Tellegen, Nicole Willems, Marianna A. Tryfonidou, Björn P. Meij

BMC Veterinary Research (2015) 7:299
Abstract

Background
Degenerative lumbosacral stenosis is a common problem in large breed dogs. For severe degenerative lumbosacral stenosis, conservative treatment is often not effective and surgical intervention remains as the last treatment option. The objective of this retrospective study was to assess the middle to long term outcome of treatment of severe degenerative lumbosacral stenosis with or without evidence of radiological discospondylitis with pedicle screw-rod fixation.

Methods
Twelve client-owned dogs with severe degenerative lumbosacral stenosis underwent pedicle screw-rod fixation of the lumbosacral junction. During long term follow-up, dogs were monitored by clinical evaluation, diagnostic imaging, force plate analysis, and by using questionnaires to owners.

Results
Clinical evaluation, force plate data, and responses to questionnaires completed by the owners showed resolution (n=8) or improvement (n=4) of clinical signs after pedicle screw-rod fixation in 12 dogs. There were no implant failures, however, no interbody vertebral bone fusion of the lumbosacral junction was observed in the follow-up period. Four dogs developed mild recurrent low back pain that could easily be controlled by pain medication and an altered exercise regime.

Conclusion
Pedicle screw-rod fixation offers a surgical treatment option for large breed dogs with severe degenerative lumbosacral stenosis with or without evidence of radiological discospondylitis in which no other treatment is available. Pedicle screw-rod fixation alone does not result in interbody vertebral bone fusion between L7 and S1.
Background

Low back pain in dogs is a common problem and can be the result of different pathologies.¹ Degenerative lumbosacral stenosis (DLSS) is the most common cause of caudal lumbar back pain in middle to large breed dogs.² DLSS is characterized by bony and soft tissue changes leading to stenosis of the spinal canal and moderate to severe compression of the cauda equina. The intervertebral disc (IVD) is often degenerated and this results in a shift of load bearing from the IVD to surrounding structures. This may lead to spinal instability. Low back pain can also be caused by other conditions, such as discospondylitis,³ trauma (fracture and/or luxation), or neoplasia.³, ⁴ Discospondylitis is a bacterial infection of the IVD and adjacent intervertebral end and commonly originates from a primary urogenital infection via hematogenous spread.³ Discospondylitis can result in severe proliferation of fibrous tissue and bone, vertebral instability, subchondral bone resorption and secondary DLSS.⁵ Computed tomography (CT) and magnetic resonance imaging (MRI) are the most informative modalities to investigate the LS area.⁶, ⁷

Treatment of DLSS can be conservative or surgical. Low back pain in DLSS can be treated with non-steroidal anti-inflammatory drugs and/or opioids, body weight reduction, and an adjusted exercise pattern or physiotherapy. Epidural infiltration with methylprednisolone acetate has been reported as medical treatment for DLSS provided that the dog do not show urinary or fecal incontinence and proprioceptive deficits, and does not suffer from concurrent discospondylitis.⁸ In case of discospondylitis long term antibiotic drugs are the primary treatment. Surgical treatment of DLSS is accomplished by dorsal laminectomy or foraminotomy, and if indicated, partial disectomy and uni- or bilateral facetectomy. In the short-term, surgical intervention leads to improvement of clinical signs in 78-93% of cases but in the long-term clinical signs recurred in 17-38% of cases,⁹, ¹⁰ which is also known as failed back syndrome.¹¹, ¹² Moreover, force plate analyses (FPAs) showed that the propulsive force of the pelvic limbs is not fully restored after decompressive surgery for DLSS.¹³ It has been postulated that decompressive surgery, and especially facetectomy, can worsen LS instability in some patients, resulting in further overall degeneration.⁹, ¹⁴

Therefore, we previously investigated the feasibility of pedicle screw-rod fixation (PSRF) in a cadaver study and in an in vivo pilot study in large breed dogs.¹⁴, ¹⁵ Screw entry points and guideline values for safe insertion of pedicle screws into the canine L7 and S1 vertebrae have been determined in other studies.¹⁴, ¹⁶, ¹⁷ The purpose of spinal fixation and interbody fusion is to restore and maintain disc space height and to increase the stability of the operated segment,¹⁸ thereby making further ongoing degenerative changes clinically irrelevant. The aim of the present study is to report the long term results of PSRF in 12 client-owned dogs with severe DLSS and also to assess whether PSRF leads to spinal fusion of the LS junction.
Materials and methods

Dogs

Twelve dogs with DLSS treated by PSRF were included in this retrospective study. The medical records of the dogs were systematically reviewed and the signalment, clinical history, findings on clinical examination, force plate data, radiographic, and CT- and/or MR imaging were retrieved. Due to the retrospective nature of the current study, no ethical approval was required. The owners consented to the use and disclosure of patient- and questionnaire data for the current study. Table 1 shows the signalment and clinical history of all the dogs included in this study.

Table 1. Overview of signalment, history and radiological diagnosis in 12 dogs with lumbosacral degenerative stenosis (DLSS) and/or discospondylitis that were treated with pedicle screw-rod fixation.

<table>
<thead>
<tr>
<th>Dog #</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>History</th>
<th>Radiological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Labrador retriever</td>
<td>FC</td>
<td>5</td>
<td>LS pain, paraparesis</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>2</td>
<td>Rottweiler</td>
<td>M</td>
<td>8</td>
<td>LS pain</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>3</td>
<td>GSD</td>
<td>FC</td>
<td>8</td>
<td>LS pain, paraparesis</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>4</td>
<td>GSD</td>
<td>MC</td>
<td>11</td>
<td>LS pain, paraparesis; DL 6 yrs earlier</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>5</td>
<td>Rhodesian ridgeback</td>
<td>F</td>
<td>10</td>
<td>LS pain, paresis left pelvic limb, urinary incontinence; DL 6 months earlier</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>6</td>
<td>GSD</td>
<td>M</td>
<td>12</td>
<td>LS pain, paraparesis</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>7</td>
<td>Cane Corso</td>
<td>MC</td>
<td>7</td>
<td>LS pain</td>
<td>DLSS</td>
</tr>
<tr>
<td>8</td>
<td>American Bulldog</td>
<td>M</td>
<td>5</td>
<td>LS pain</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>9</td>
<td>Border Collie</td>
<td>MC</td>
<td>9</td>
<td>LS pain</td>
<td>DLSS &amp; DS</td>
</tr>
<tr>
<td>10</td>
<td>Rhodesian Ridgeback</td>
<td>FC</td>
<td>7</td>
<td>LS pain, paraparesis; DL 4 yrs earlier</td>
<td>DLSS</td>
</tr>
<tr>
<td>11</td>
<td>Vizsla</td>
<td>MC</td>
<td>12</td>
<td>LS pain</td>
<td>DLSS</td>
</tr>
<tr>
<td>12</td>
<td>Am. staff. terrier</td>
<td>F</td>
<td>5</td>
<td>LS pain, left paresis pelvic limb, DL 3 yrs earlier</td>
<td>DLSS</td>
</tr>
</tbody>
</table>

Am. staff. terrier = American Staffordshire terrier, DS = discospondylitis, DL = dorsal laminectomy, F = female, FC = female castrated, GSD = German Shepherd Dog, LS = lumbosacral, M = male, MC = male castrate, yrs = years.

Clinical examination

All dogs underwent a full clinical examination, consisting of a general physical, orthopedic and neurological examination by a board-certified veterinary surgeon (BPM). Neurological deficits were graded based on the scale used by Griffith (modified by Sharp and Wheeler 2005): grade 0 (normal), grade 1 (spinal pain only), grade 2 (ambulatory paraparesis), grade 3 (non-ambulatory paraparesis), grade 4 (paraparesis with deep pain perception), and grade 5 (paraparalysis without deep pain perception) (Table 2).

Diagnostic imaging

Ventrodorsal and lateral radiographic views were obtained with the LS spine in neutral position. CT- and MRI-scans were obtained under general anesthesia and dogs were
positioned in sternal recumbency with the pelvic limbs extended caudally. CT-scans were obtained with a third-generation CT-scanner (Tomoscan CX/S, Philips). Contiguous 2-mm-thick slices were acquired. MRI was performed with a 0.2 Tesla open magnet (Magnetom Open Viva, Siemens). Contiguous 3-mm-thick sagittal T1- and T2-weighted images and transverse T1-weighted MR images were obtained.

Pre-operatively, CT-scans and/or MRI scans were performed. The acquired diagnostic images were evaluated by a board-certified radiologist, a board-certified orthopedic surgeon (BPM), and a PhD student/DVM (ART). During surgery, correct position of the screws and the amount of distraction was verified by fluoroscopy. Post-operatively, the position of the pedicle screws, the amount of bony fusion and the development of adjacent segment pathology (ASP) were recorded by radiography or computed tomography on several occasions. In four dogs manual distraction was applied, and the amount of distraction was calculated by comparing the disc height indices prior to treatment with the PSRF device in place. The disc height index was calculated on the radiographs and midsagittal CT reconstructions by using the method described by Hoogendoorn et al. Imaging performed during follow up visits is summarized in Table 3.

**Force plate analysis (FPA)**

Ground reaction forces (GRFs) were measured using a quartz crystal piezoelectric force plate (Kistler type 9261, Kistler Instrumente) together with the Kistler 9865E charge amplifiers. The force plate itself was 60 cm wide and 40 cm long, and was mounted flush with the surface in the center of an 11 m long walkway. The middle 5 m of the runway was bordered by a 50-cm high fence to guide the dogs over the force plate. GRFs were measured by force transducers, which were located in every corner of the plate. The amplifiers were connected to an analog-digital converter, interfaced with a computer that stored the signals. The sampling rate was 100 Hz. The signals corresponded with the GRFs in the mediolateral (Fx), craniocaudal (Fy) and vertical (Fz) direction. The Fz was calibrated with a standard weight before each recording session. Forward velocity of the dog was measured during FPA, using two photoelectric switches spaced 3 m apart and centered on the force plate and computer timing. FPA recordings were automatically started and stopped by these photoelectric switches. All dogs were guided over the force plate on a leash at a walking gait with an average speed of 1.08 m/s (standard deviation 0.08 m/s). Data recorded from measurements in which a thoracic limb and, after a short interval, the ipsilateral pelvic limb contacted the plate were considered valid. A minimum of eight recordings were used for data processing. All forces were normalized for body weight. Ratios between pelvic (P) and thoracic (T) limbs were calculated: P/T Fy, P/T Fy⁺ and P/T Fz⁺. Obtained results were compared to previous FP results in normal dogs and dogs with
low back pain.19

Surgical procedure and postoperative care

All dogs were operated by the same ECVS board-certified surgeon (BPM). All dogs underwent a dorsal laminectomy2 and several additional procedures before PSRF depending on the imaging and surgical findings (Table 2). Discectomy yielded nucleus pulposus (NP) material that was cultured for bacteria in 10/12 dogs. The spinous processes of L7 and S1 and the lamina of L7 were preserved to serve as autologous bone transplant in ten dogs. In one dog (case 4) a cancellous bone transplant was obtained from the iliac crest. The bone chips and cancellous bone were packed into the intervertebral disc space up to 5 mm beneath the floor of the vertebral canal. An autologous fat transplant, harvested from free subcutaneous tissue, was placed ventral to the cauda equina, and a larger piece was deposited dorsally in the laminectomy site with the aim of preventing dural adhesions and new bone formation.2 In one dog the compression was severely lateralized necessitating a unilateral facetectomy (dog 5), in two other dogs bilateral facetectomy was necessary (dog 8 and 10). In dog 5 the left S1 nerve had an abnormal appearance and was completely resected and sent for histology.

Thereafter, PSRF was performed as described by Smolders et al.14 Briefly, the entry points of L7 and S1 were identified and the corridors in the cancellous bone within the pedicle were prepared using a bone awl and probe (USS Small Stature, Synthes). Once the ventral cortex was reached, the pedicle probe was removed from the screw corridor. To facilitate screw anchorage in the ventral vertebral cortex, predrilling of the ventral cortex was performed with a K-pin (1.2 mm). Four 25 mm long, 4 mm wide titanium pedicle screws4 were inserted into the pedicle and vertebral body. Two 5 cm long, 6 mm wide titanium rods were used to connect the L7 pedicle screw with the ipsilateral S1 pedicle screw. The rod was slightly adjusted with a rod bender to acquire a proper fit on both screw heads. Once a tight fit was obtained, the sleeves and nuts were applied and tightened. Optimal screw anchorage was achieved by involving both the medial and lateral pedicle cortex. “Cortical encroachment” was identified when the pedicle cortex could not be visualized or as “frank penetration” when the screw was outside the pedicular boundaries.59-61 Screw placement was considered optimal when screws involved the cortical bone and not fully penetrated the ventral vertebral cortex. Intraoperative fluoroscopy was used to verify correct placement of the screws. Four dogs underwent manual distraction as well because of intervertebral foraminal stenosis evident on pre-operative imaging. Manual distraction was applied to the base of the pedicle screws using a Gelpi retractor followed by tightening the screw heads to the rods. The amount of distraction was estimated based on the mobility of the LS segment and did not exceed 5 mm. Postoperative care consisted of leash restraint and exercise restriction for a period of six weeks and after that, the dogs
were allowed to gradually return to their normal exercise regime within three months after surgery.

**Follow-up and questionnaires to owners**

Follow-up data were collected from the medical records, by using questionnaires to owners, by interviewing the owners and by reexamination of the dogs. Questionnaires for follow-up evaluation (Table 5) were sent to all owners of dogs that had undergone PSRF within the last four years. Two dogs were lost in follow-up due to unrelated mortalities. The questionnaires included questions regarding the history, clinical signs before surgery and the owner’s satisfaction with the outcome at three months and one year after surgery.

**Table 5.** Questionnaire to the owners of dogs before, at three months and more than one year after pedicle screw-rod fixation for degenerative lumbosacral stenosis.

<table>
<thead>
<tr>
<th>Types</th>
<th>Questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>YES or NO questions</td>
<td>Did the symptoms disappear after surgery?</td>
</tr>
<tr>
<td></td>
<td>Did the symptoms recur after surgery (after initial improvement)?</td>
</tr>
<tr>
<td>Open questions</td>
<td>How is your dog after surgery?</td>
</tr>
<tr>
<td></td>
<td>Does your dog refuse certain movements?</td>
</tr>
<tr>
<td></td>
<td>Did your dog receive further treatment after surgery?</td>
</tr>
<tr>
<td>Questions with a 10-point scale</td>
<td>Does your dog have pain in the pelvic limbs and shows lameness?</td>
</tr>
<tr>
<td></td>
<td>Does your dog show weakness in the pelvic limbs?</td>
</tr>
<tr>
<td></td>
<td>Does your dog have low back pain?</td>
</tr>
<tr>
<td></td>
<td>Does your dog have difficulty rising up?</td>
</tr>
<tr>
<td></td>
<td>Does your dog have difficulty lying down?</td>
</tr>
<tr>
<td></td>
<td>How would you rate muscle volume in the pelvic limbs of your dog?</td>
</tr>
<tr>
<td></td>
<td>How is your dog holding its tail?</td>
</tr>
<tr>
<td></td>
<td>Is your dog able to wag its tail?</td>
</tr>
<tr>
<td></td>
<td>Does your dog show loss of control of urination and defecation?</td>
</tr>
<tr>
<td></td>
<td>Does your dog show pain when you touch the lower back?</td>
</tr>
</tbody>
</table>

**Statistical analysis**

Statistical analysis was performed using software (SPSS 22 for Windows; SPSS Inc., Chicago, IL). Normal distribution of the data was checked by performing the Shapiro Wilks test. The reliability of the responses to the questionnaires was tested by calculation of Cronbach’s α where a value of >0.70 was considered reliable. Comparison of the mean scores of the questionnaires before surgery, at 6 months, and >1 years after surgery was conducted using the Friedman’s test. If there was a significant difference (P<0.05), post hoc tests were performed for each time point. The pre-operative Griffith score for neurological (dys)function was compared to the Griffith score appointed at the last follow up visit using the Wilcoxon signed rank test. Significance was set at P<0.05.
Chapter 6

Results

Dogs

Seven male (3 intact, 4 neutered) and five female (2 intact, 3 neutered) dogs with a median age of 8 years (1 – 12 years) and a median body weight of 32 kg (22 – 55 kg) were included in the study (Table 1). All dogs were kept as companion animals. Four dogs had undergone previous decompressive surgery once but developed failed back syndrome.

Clinical examination

All dogs presented with pelvic limb lameness and caudal lumbar pain; seven dogs also showed paraparesis. In all dogs pain was evoked upon pressure and extension of the LS spine and tail extension. One dog suffered from urinary incontinence. The neurological Griffith score before surgery was grade 1 (5 dogs), 2 (4 dogs) and 3 (3 dogs) (Table 2).

Table 2. Overview of surgery details and clinical outcome in 12 dogs with lumbosacral degenerative stenosis (DLSS) that were treated with pedicle screw-rod fixation. Excellent: resolution of clinical signs. Improved: decrease of clinical signs.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Surgery</th>
<th>Bone graft</th>
<th>Clinical outcome (follow-up (FU) period)</th>
<th>Griffith score pre-op</th>
<th>last FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L7-S1: DL, PD, PSRF</td>
<td>Bone L7+S1</td>
<td>Excellent (4 yrs)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>L7-S1: DL, PD, PSRF</td>
<td>Bone L7+S1</td>
<td>Excellent (4 yrs)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>L7-S1: DL, PD, PSRF</td>
<td>Bone L7+S1</td>
<td>Excellent (3 yrs)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>L7-S1: rDL, PD, PSRF &amp; Distraction</td>
<td>Bone iliac crest, Osteostixis EPs</td>
<td>Improved (euth. 6 mo heart disease)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>L7-S1: rDL, PD, L Facetectomy, L Foraminotomy, Excision L7 nerve, PSRF</td>
<td>Bone L7+S1</td>
<td>Improved (euth. 15 mo, neoplasia)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>L6-S1: DL, L7-S1: DL, PSRF</td>
<td>Bone L7+S1</td>
<td>Improved (1.5 years, euth. hemangiosarcoma)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>L7-S1: DL, PD, L&amp;R Facetectomy, PSRF &amp; Distraction</td>
<td>Bone L7+S1</td>
<td>Excellent (1 yr)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>L7-S1: DL, PD, PSRF</td>
<td>Bone L7+S1</td>
<td>Improved (euth. 8 mo)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>L6-S1: DL, L7-S1: PD; L&amp;R Facetectomy, PSRF &amp; Distraction</td>
<td>Bone L7+S1</td>
<td>Excellent (6 mo)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>L7-S1: rDL, PD, PSRF</td>
<td>Bone L7+S1</td>
<td>Excellent (6 mo)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>L6-S1: DL, L7-S1: PD PSRF &amp; Distraction</td>
<td>Bone L7+S1</td>
<td>Excellent (11 mo)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>rDL, Partial L Facetectomy, L Foraminotomy, PSRF</td>
<td>None</td>
<td>Excellent (6 mo)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

DL = dorsal laminectomy, rDL = revision DL, PD = partial discectomy, PSRF = pedicle screw-rod fixation, L = left, R = right, EPs = endplates, yrs = years, mo = months, euth. = euthanized, FU = follow-up
Diagnostic imaging

Imaging was performed pre-operatively with plain radiography (4 dogs), CT (12 dogs), and MRI (5 dogs) (Table 3). In all 12 dogs the final radiological diagnosis was DLSS with presumptive radiologic evidence of concurrent discospondylitis in eight dogs (Table 3). Pre-operative radiologic and CT findings included spinal stenosis of the lumbosacral junction (Figure 2a) in ten dogs, end plate sclerosis of both lumbosacral end plates (Figure 3) in eleven cases, end plate osteolysis (Figure 1) in seven cases, vacuum phenomenon in the IVD (Figure 2a) in three cases, elongation of the sacral lamina up to or under the caudal end of the lamina of L7 as described by Suwankong et al (Figure 1) in four cases and LS step formation (ventral subluxation of S1 with respect to L7) (Figure 3) in four cases. A narrowed IVD space was visible in two dogs. Non-bridging spondylosis deformans (Figure 3) was recorded pre-operatively in nine dogs, bridging spondylosis in two dogs. Protrusion of the IVD was seen in all dogs; severe protrusion (>50% reduction of spinal canal width) (Figure 1a) in ten dogs, a moderate compression (25-50% reduction of spinal canal width) in one dog and mild protrusion (<25% reduction of spinal canal width) in one dog. Dorsal displacement of the dural sac, combined with a decrease in the epidural fat signal dorsal to the dural sac at the level of L7-S1 was recorded in nine dogs on MRI or CT (Figure 1). Thickening of spinal nerves was detected in four dogs. The signal intensity of the L7-S1 IVD on T2-weighted images was severely decreased in all five dogs which underwent MRI (Figure 1). Dog 4 had undergone dorsal laminectomy 6 years earlier (Table 1) and on MR a bulging LS disc was noted in combination with dorsal displacement of nerve tissue at the level of L7-S1. Calcifications in the IVD space were recorded as well. On the CT images there was marked ventral spondylosis deformans, IVD calcifications and vacuum phenomenon. Moreover, there was still severe central and right lateral disc protrusion present, leading to the right lateral nerve compression near the right facet joint. Dog 5 had undergone decompressive surgery 6 months earlier (Table 1). CT showed that the cauda equina was displaced dorsally as a consequence of bulging disc material. Both the L7 and S1 end plates were irregular and sclerotic. There was pronounced new bone formation around the lumbosacral junction, in the intervertebral foramina and around the sacroiliac joints. The left exiting spinal nerve was markedly enlarged, indicative for a peripheral nerve sheath tumor. There was severe muscle atrophy present in the left quadriceps and gluteus muscles.
Table 3. Overview of read out parameters in 12 dogs with lumbosacral degenerative stenosis (DLSS) treated with pedicle screw-rod fixation.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Pre-op</th>
<th>Intra-op</th>
<th>Post-op</th>
<th>Follow up period (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 3 mo</td>
<td>&lt; 6 mo</td>
</tr>
<tr>
<td>1</td>
<td>CT, MRI</td>
<td>BC, HP</td>
<td>RX</td>
<td>CT</td>
</tr>
<tr>
<td>2</td>
<td>RX, CT</td>
<td>BC, HP</td>
<td>RX</td>
<td>RX</td>
</tr>
<tr>
<td>3</td>
<td>RX, CT</td>
<td>BC, HP</td>
<td>RX</td>
<td>RX</td>
</tr>
<tr>
<td>4</td>
<td>CT, MRI</td>
<td>BC, HP</td>
<td>RX, CT</td>
<td>RX</td>
</tr>
<tr>
<td>5</td>
<td>CT, MRI</td>
<td>BC, HP</td>
<td>RX</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>RX, CT</td>
<td>BC</td>
<td>RX</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CT, MRI</td>
<td>BC</td>
<td>RX</td>
<td>CT, FPA (10 mo)</td>
</tr>
<tr>
<td>8</td>
<td>CT</td>
<td>BC</td>
<td>RX</td>
<td>CT, RX, FPA</td>
</tr>
<tr>
<td>9</td>
<td>CT, MRI</td>
<td>BC, HP</td>
<td>RX</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CT, MRI</td>
<td>BC, HP</td>
<td>RX</td>
<td>CT, FPA</td>
</tr>
<tr>
<td>11</td>
<td>CT, RX, MRI</td>
<td>BC, HP</td>
<td>RX</td>
<td>RX</td>
</tr>
<tr>
<td>12</td>
<td>CT</td>
<td>BC, HP</td>
<td>RX</td>
<td>RX</td>
</tr>
</tbody>
</table>

Pre-op = pre-operative, intra-op = intra-operative, post-op = postoperative, mo = months, CT = computed tomography, MRI = magnetic resonance imaging, BC = bacteriologic culture, HP = histopathologic evaluation, RX = plain radiography, FPA = force plate analysis

**Figure 1A.** Sagittal T2-weighted MR image of a 5-year-old Labrador retriever (dog 1) with degenerative lumbosacral stenosis and acute onset of discospondylitis. There is a hyperintense signal (exudate) visible in the intervertebral disc space. **1B.** Sagittal T2-weighted MR image of dog 1 after three months of treatment with oral antibiotics. The inflammatory exudate has disappeared. **1C.** Immediate postoperative radiograph of dog 1 after pedicle screw-rod fixation (PSRF) showing osteolysis of the L7 and S1 endplates. **1D.** Radiograph of dog 1 at four years after PSRF. Spondylosis deformans has formed ventral to the LS junction.

**Figure 2A.** Transverse CT image of the lumbosacral (LS) junction of a 9-year-old Border collie (dog 9) with degenerative lumbosacral stenosis and discospondylitis. Spinal stenosis and severe intervertebral disc (IVD) bulging are visible and there is gas accumulation (vacuum phenomenon) present in the center of the L7-S1 IVD. **2B.** Transverse CT image at level of S1 of a dog (dog 1) with pedicle screw-rod fixation, four years after implantation.

No bony fusion between the L7 and S1 vertebrae was visible.
Surgical findings
Following dorsal laminectomy and partial discectomy (Table 2), pedicle screws were inserted and were used to distract, realign and stabilize the LS segment. In ten dogs, the protrusion of the IVD was considered severe, in one dog, there was moderate protrusion. The amount of epidural fat was decreased in ten dogs and absent in one dog. In six dogs, inflammation of the epidural fat was noticed by the surgeon. In 11 dogs thickening of neural tissue, especially the S1 nerve roots, was visible. Two of ten disc tissue samples returned with a positive bacterial culture. *Bacillus spp* (dog 5) and *Staphylococcus aureus* (dog 8) were identified in two dogs. Histopathological examination of tissue samples collected during surgery showed degeneration of the annulus fibrosus and nucleus pulposus in all cases. Histopathological examination of the excised nerve (dog 5) showed an undifferentiated neurofibrosarcoma of the nerve root, characterized by round- and spindle shaped neoplastic cells.

Follow-up (imaging and clinical signs)
Radiography or CT was performed to evaluate the position of the screws and the amount of interbody vertebral bone fusion. In the follow-up period after surgery imaging was performed at 4 – 6 weeks (radiography or CT, 7 dogs), at three months (radiography or CT, 4 dogs), at six months (CT, 3 dogs), at one year (CT, 2 dogs), at three years (radiography or CT, 2 dogs), and at four years (CT, 1 dog) (Table 2). Placement of the screws was considered to be correct in 11 out of 12 dogs (92%) based on radiographic evaluation. In six dogs, CT was performed postoperatively (Figure 1b). Optimal screw anchorage was achieved by involving both the medial and lateral pedicle cortex. Cortical encroachment of
the lateral pedicle wall was noticed on CT with the right L7 screw in two dogs. Penetration of the ventral cortex was recorded on CT in three dogs, involving four screws. No implant failures were seen.

In eight dogs, there was complete resolution of clinical signs after surgery, in two dogs the severity of the clinical signs decreased. These two dogs (dog 4 and 5) had already undergone prior decompressive surgery by dorsal laminectomy. In two dogs (dog 6 and 8), the clinical signs recurred after initial remission. Plain radiographs and CT scans were obtained. No adverse advents as a result of the pedicle screw implantation surgery were noted. Neurologic dysfunction in dog 6 did not improve markedly after surgery and dragging with the left hind limb persisted. Dog 8 was euthanized at eight months after surgery at request of the owner, since low back pain recurred every time antibiotic treatment was ceased. After surgery, the Griffith neurological grading score was 0 (9 dogs), 1 (1 dog), 2 (1 dog) and 3 (1 dog) (Table 1). The median pre-operative Griffith score was 2 (with a range from 1-3), whereas the Griffith score obtained at the last follow up visit was 0 (with a range from 0 to 3). The Wilcoxon signed rank test revealed a significant improvement in Griffith scores before surgery and at the last follow up visit (p=0.004).

The development of adjacent segment pathology was noticed in one dog after three years on plain radiographs (Figure 3), but the dog did not display signs of low back pain. At any time point after PSRF, in none of the other dogs ASP was noticed on diagnostic imaging nor clinically. In the four dogs that underwent manual distraction of the LS junction, the IVD space height increased by 67% (dog 4), 11% (dog 7), 114% (dog 9), and 9% (dog 11) compared with the IVD height prior to surgery. Six months after surgery, distraction of the LS junction was still present in three dogs. In one dog (dog 11) there was loss of distraction as evidenced by sudden low back pain at one week postoperatively, and radiographic evidence of collapse of the L7-S1 IVD space without implant failure. The pain was controlled with oral analgesics for two weeks.

**Force plate analysis**
Pre-operative force plate analysis was performed in three dogs (dogs 8, 10 and 12) (Figure 4). In two dogs (dogs 8 and 10), the P/T Fy and P/T Fz ratios were lower than reference ranges described in a previous study. In dog 10, FPA was performed six months after surgery and values were still below reference ranges, this dog was lost for further follow up. Dog 7 showed normal P/T Fy values after 10 months. FPA performed in three dogs (dog 1, 2 and 3) more than three years after surgery showed P/T Fy ratios comparable to normal dogs.
Owner questionnaires

Eight out of twelve (67%) owners responded to the questionnaire. The follow-up period ranged from five months to more than four years. Prior to surgery, owners mentioned low back pain, hind limb lameness, and reluctance to perform certain movements as the most striking clinical signs. All owners reported that the clinical signs of their dog had disappeared after surgery. However, in four dogs the clinical signs recurred. In three dogs these signs were mild and could be treated effectively with NSAIDs. In the fourth dog, discospondylitis persisted despite aggressive long term (6 months) antibiotic therapy, for which the dog was eventually euthanized at eight months after PSRF. Six owners did not report any recurrences during the follow-up period; the long term outcome of three dogs is unknown since they were euthanized due to unrelated illnesses (i.e. heart failure, hemangiosarcoma). Three dogs showed concurrent orthopedic problems such as hip dysplasia \((n=1)\) and osteoarthritis of the stifle joint \((n=2)\) in the follow up period. Five dogs continued to receive intermittent pain medication, with four dogs receiving non-steroidal anti-inflammatory drugs (NSAIDs), one dog tramadol and one dog a combination of NSAIDs and neuromodulatory drugs (gabapentin).

Before evaluating the answers of the owners to the questionnaire, the reliability of the answers was tested by calculating the Cronbach’s alpha value. The Cronbach’s alpha value of the responses to the questionnaire was 0.88, indicating that the answers were reliable. The data were normally distributed. All eight owners that had filled in the questionnaire reported that the clinical signs of low back pain had disappeared after surgery (100%). Three owners reported that the clinical signs of low back pain had recurred after an asymptomatic period of time \((3/8 = 38\%)\). Table 4 shows the results to the questionnaire before, six months, and more than one year after surgery. All data are expressed as the median and the range. The level of significance was set at \(P <0.05\). There was a significant and sustained decrease in caudal limb lameness, caudal lumbar pain and difficulty in rising up more than six months after surgery. Moreover, muscle volume had significantly
increased six months after surgery, compared to the pre-operative situation. There was a trend of decrease in pelvic hind limb lameness and hypersensitivity of the caudal spine (P=0.061) after six months.

Table 4. Results (median and range) of responses to the questionnaires of dogs treated with PSRF before surgery, after 6 months and more than 1 year after surgery.

<table>
<thead>
<tr>
<th>Questions</th>
<th>Before surgery</th>
<th>After 6 months</th>
<th>After &gt; 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complaints of pelvic limbs</td>
<td>3 (1-4)</td>
<td>8 (4-10) *</td>
<td>7 (5-9) *</td>
</tr>
<tr>
<td>Pelvic limb weakness</td>
<td>4 (1-10)</td>
<td>8 (6-10) †</td>
<td>7 (6-10) †</td>
</tr>
<tr>
<td>Caudal lumbar pain</td>
<td>1 (1-4)</td>
<td>7 (4-10) *</td>
<td>7 (5-9) *</td>
</tr>
<tr>
<td>Difficulty rising up</td>
<td>4 (1-6)</td>
<td>8 (7-10) *</td>
<td>7 (5-10) *</td>
</tr>
<tr>
<td>Difficulty lying down</td>
<td>8 (1-6)</td>
<td>10 (8-10)</td>
<td>10 (5-10)</td>
</tr>
<tr>
<td>Muscle volume of the pelvic limbs</td>
<td>4 (1-7)</td>
<td>7 (5-9) *</td>
<td>7 (6-7) *</td>
</tr>
<tr>
<td>Position of the tail</td>
<td>3 (1-10)</td>
<td>9 (1-10)</td>
<td>9 (1-10)</td>
</tr>
<tr>
<td>Movement of the tail</td>
<td>5 (2-10)</td>
<td>10 (3-10)</td>
<td>9 (3-10)</td>
</tr>
<tr>
<td>Control of urination and defecation</td>
<td>10 (3-10)</td>
<td>10 (10-10)</td>
<td>10 (10-10)</td>
</tr>
<tr>
<td>Hypersensitivity of the caudal spine</td>
<td>3 (1-10)</td>
<td>9 (3-10) †</td>
<td>10 (9-10)</td>
</tr>
</tbody>
</table>

* P < 0.05, Friedman test, compared with the value before surgery. † Group comparisons were borderline significant (P=.061), individual comparisons of time points did show a significant difference (P<0.05)

Discussion

The pilot study of Smolders et al (2012) suggested that PSRF of the canine LS junction can be used as an addition to surgical decompression for dogs with LS disease and presumed instability of the LS joint. The results showed stability of the implants and improvement of hind limb function in Greyhounds with mild LS disease. The current study presented the follow-up of 12 client-owned dogs with severe DLSS treated with PSRF. With data retrieved from diagnostic imaging, FPAs and clinical examinations together with owner questionnaires, we conclude that PSRF can be a feasible treatment option for dogs with DLSS in which previous decompressive surgery failed and/or medical treatment is ineffective to control low back pain. The authors are aware of the limitations of this retrospective study. The study group is relatively small and due to the retrospective nature of the study, the follow up of the patients was not standardized. Not all owners were willing to attend control visits with their dog or had financial constraints. All cases were referred as severe and complicated cases, where conservative treatment or previous surgery had failed, or for which no other treatment was available. Even more, in several cases euthanasia was advised by the referring veterinarian but the owner persisted for third opinion referral.

Propulsive forces in the hind limb are decreased in dogs with DLSS as compared to healthy dogs. In the present study, the collected FPA data showed an initial worsening after surgery, but after six months overall results were improving, with values at six months
after surgery higher than before surgery. Notably, ground reaction forces were comparable to normal dogs (table 3). These findings are in agreement with results from previous studies on FPA before and after decompressive surgery and the in vivo pilot study on PSRF from the same group. Given that FPA is used to objectively measure ground reaction forces in both humans and dogs, these findings indicate an overall clinical improvement in the long term.

The percentages of dogs with clinical remission and recurrence found in our study were similar to those for dorsal laminectomy alone, although the dogs in the current study suffered from more severe LS disease than the average population. Decompressive surgery has proven to be insufficient in a small percentage of cases, i.e., due to the development or worsening of LS instability after surgery. In four dogs in this study a previous decompressive surgery was already performed with inadequate effect. In humans with low back pain due to end stage degenerative disc disease, spinal fusion using cages with or without pedicle screw fixation is currently the state-of-the-art, rather than decompressive surgery alone. Moreover, spinal fusion is often performed during revision surgery for failed back syndrome.

Placement of the screws was considered to be correct in 48/52 screws (92%) and no implant failures were seen. In three cases, cortical encroachment of the medial pedicle wall by four screws was detected but this did not result in clinical signs. Optimal screw anchorage is achieved by involving the cis- and trans-cortex, as well as the medial and lateral pedicle wall. Full penetration of the ventral cortex was seen with seven screws in five dogs. Although full penetration carries the risk of damaging vascular structures, there was no indication that this happened. Full penetration is most likely the result of the fixed length of the screws. The pedicle screw rod fixation device that was used in this study was produced for paediatric human spinal fixation which apparently was still too large for some of the dogs, e.g. dog number 11 (Border collie). This underscores the need for the development and production of pedicle screws for the canine species.

The aim of PSRF is to stabilize the LS junction. This is achieved in the short term by the inserted instrumentation and in the long term by fusion of the spinal segments. However, in this study no interbody vertebral bone fusion was achieved. Several authors have reported on surgical stabilization in the veterinary field as well. McKee et al. have performed distraction-stabilization in dogs with discospondylitis by the method described by Slocum et al. and Auger et al. have performed articular facet joint distraction with an external fixator. More recently, Golini et al published a study about transarticular fixation as treatment for DLSS in dogs. In all abovementioned studies, a considerable number of implant failures was seen which in some cases required additional surgery. In
In the current study, there was no implant failure. The dogs recovered very well but there was no evidence for spinal fusion in the long term follow-up. To achieve interbody fusion, additional methods are necessary. In the current study, we used autologous bone grafts in 11 cases but without success as far as bony fusion is concerned. Fitzpatrick and colleagues developed a dorsal fixation system, which uses a screw-rod construct in combination with a wedge-shaped screw. This screw is positioned between the L7 and S1 vertebrae. With this device, bone ingrowth was visible. In human medicine, interbody spinal fusion is promoted by several techniques. In addition to iliac crest autograft, metal and composite interbody cages, allograft bone dowels and bone grafts infused with recombinant bone morphogenetic proteins (BMPs) or bone marrow derived stem cells are readily available for human patients and show promising effects. Moreover, in dogs the subchondral bone is relatively thicker than in humans whereas the canine end plates are thinner. This may counteract bony fusion between the two vertebrae in canines. Therefore more aggressive burring of the end plates to penetrate the subchondral bone would be appropriate in canines to achieve spinal fusion. Although bony fusion of the last lumbar vertebra and the sacrum is desired, there was no significant difference in outcome in human and canine patients that did show spinal fusion compared to patients that failed to develop interbody fusion after spinal fusion surgery.

Recurrence of clinical signs after PSRF stabilization could be related to adjacent segment pathology (ASP). ASP can be defined as degeneration or other pathologic processes occurring cranial or caudal to a region of vertebral column fusion, the most common pathology being IVD degeneration. ASP has been found in humans after spinal fusion surgery and also in dogs after cervical fusion. Lumbar spinal fusion in humans resulted in radiologic evidence of ASP in 10-80% after 10 years. Loss of motion in the fused segment leads to increased workload and altered biomechanics in adjacent segments. However, at this moment it is unclear if ASP is a natural degenerative process or if ASP is the result of fusion surgery. Clinically relevant ASP was only noted in 6 – 26.1% of the human patients, with radiologic confirmed ASP, after ten years. ASP does not seem a frequent clinical problem in dogs, most likely since they may not live long enough to develop adjacent segment pathology. In humans, the increasing number of fused vertebrae is associated with an increased risk of developing ASP. Additionally, dorsal laminectomy adjacent performed to the fused segment, pre-existent IVD degeneration and pre-existent facet degeneration in the adjacent segment are also risk factors associated with the development of clinical ASP.

Only two of the ten bacterial cultures showed a positive result, even though in eight dogs, there was radiological evidence for discospondylitis. It remains also unclear whether in these eight dogs discospondylitis was the primary etiology or whether it was
superimposed on pre-existent DLSS since the end stage of severe lumbosacral discospondylitis is usually DLSS. Making the definitive diagnosis of discospondylitis is also challenging, for the detection of bacteria in the IVD can be rather difficult. Extensive degenerative changes in the IVD could also resemble discospondylitis. Urine and blood cultures only give positive results in 29 to 78% of the cases\textsuperscript{44, 45} and due to antibiotic treatment prior to culture, bacterial cultures often remain negative.\textsuperscript{45, 46} This could also be the case in our study, as five dogs were treated for discospondylitis conservatively with antibiotics prior to the collection of disc material for bacterial culture. Interestingly, the topic of bacteria in IVDs causing low back pain has received considerable attention in recent years in the field of spine research in humans and has since been the subject of heated debate.\textsuperscript{47-49} This debate was initiated by reports by Albert et al.\textsuperscript{50} on findings of bacteria in IVD material harvested during spinal surgery\textsuperscript{51} and publication of a randomized clinical trial showing successful treatment of humans with chronic low back pain using long term oral antibiotics.\textsuperscript{52} In the light of these findings in humans, the positive bacterial cultures in our canine patients with low back pain which has been reported by our group previously\textsuperscript{6} are not surprising. It may even be questioned whether the environment of the degenerated IVD in dogs with DLSS is more prone to settling of bacteria originating from low grade urogenital infections or that bacteria indeed play a much more important role as the initiating factor in the process of IVD degeneration in dogs.

Distraction of the IVD space results in widening of the foramina and thereby results in indirect decompression of the exiting L7 spinal nerves, it will limit motion and permit fusion.\textsuperscript{18} Moreover, distraction can normalize disc height and pressure.\textsuperscript{53} A combination of spinal fixation through PSRF and distraction without concurrent discectomy could potentially show a beneficial effect on stability and IVD physiology in dogs, as is seen in human patients suffering from end stage knee osteoarthritis. After two months of applied distraction of the knee joint, clinical improvement and the formation of cartilage-like tissue in the distracted knee were evident for at least two years.\textsuperscript{54} In this study, PSRF in combination with discectomy and distraction was performed in four dogs. Postoperative radiography showed successful distraction in all four cases. In three dogs the LS joint remained distracted for at least six months postoperatively. The fourth dog became very painful three days after surgery and radiography showed collapse of the L7-S1 IVD space. The dog was treated with pain medication and clinical signs resolved. This case demonstrates that distraction alone with PSRF in dogs with severe DLSS exerts strain on the interface between bone and pedicle screws and this may be solved by the use of an vertebral interbody cage. In dogs with caudal cervical spondylomyelopathy, a combination of vertebral stabilization and intervertebral implants tend to be more effective in gaining bony fusion and can also maintain distraction.\textsuperscript{39} In spinal surgery in human patients with low back pain, intervertebral cages are also frequently used (with or without vertebral
Aggressive abrasion of the end plates together with a spinal cage may also promote spinal fusion. The use of a cage as a stand-alone-device or in combination with PSRF (and the effect on spinal fusion) needs to be investigated in future studies.

Conclusion
PSRF can be an effective therapy option for dogs with severe DLSS disease with or without radiological evidence of discospondylitis, in which no other treatment is available. PSRF alone does not result in interbody vertebral bone fusion between L7 and S1.

Acknowledgements
We would like to thank Arie Doornenbal and Elise Petersen for their technical assistance.
References


Chapter 7

Temporary segmental distraction as a treatment for a dog with degenerative lumbosacral stenosis

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Manuscript in preparation
Abstract

Introduction
Degenerative lumbosacral stenosis (DLSS) causing low back pain is a common disorder in medium and large breed dogs, in which intervertebral (IVD) degeneration and herniation (Hansen type II) play an important role. The pathogenesis, biomechanics, clinical signs and therapy of lumbar IVD herniation are similar between humans and dogs. Current treatments in both species aim at alleviating symptoms, but do not result in regeneration of the degenerated IVD. Temporary distraction in rabbit models with induced IVD degeneration, showed signs of IVD repair at a biological, cellular, and biomechanical level. In this study, we assessed the safety and efficiency of temporary segmental distraction in a dog with clinical signs of DLSS that was unresponsive to conservative management.

Methods
Temporary (3 months) distraction of the lumbosacral (LS) junction by pedicle screw-rod fixation (PSRF) was applied in a 5-year-old Greyhound and evaluated by radiography (disc height index and IVD area), MR imaging (T2-weighted images and T2 mapping values), and force plate analysis at several time points: before (t-1), directly after application of the distraction device (t0), after 3 months of distraction at removal of the device (t3), and again at 6 (t6) and 9 months (t9) follow-up.

Results
Safe temporary distraction of the LS junction was demonstrated by using PSRF, with improvement of clinical signs over the course of 6 months after removal of the distraction device. Signal intensity of the IVD showed no changes over time. T2 value was highest directly after removal of the distraction device, but decreased by 10% of the pre-operative value at 9 months follow-up. Disc height was slightly decreased (8%) immediately after removal of the distraction device, but recovered to the initial value at 9 months follow-up. An initial decrease of the pelvic/thoracic propulsive force (P/T Fy-) during PSRF + distraction was demonstrated, which slowly increased after removal of the distraction device to a value at 9 months follow up that was 4% higher than the initial preoperative P/T Fy- value.

Conclusion
Temporary PSRF in combination with distraction in this patient was shown to be safe, to improve clinical signs and to retain disc height at 9 months follow-up. In order to evaluate the safety and clinical efficacy of this technique in the treatment of DLSS and regeneration of the IVD, more patients need to be included in future studies, and longer follow-up times need to be considered.
Introduction

Degenerative lumbosacral stenosis (DLSS) is a common disorder in medium and large breed dogs. It is a disorder of multifactorial origin, in which IVD degeneration and herniation (Hansen type II) play an important role. In IVD degeneration, the delicate equilibrium of the extracellular matrix (ECM) synthesis and degradation in the gelatinous nucleus pulposus (NP), the surrounding fibrous annulus fibrosus (AF), and the cartilaginous endplates (EPs) shifts toward the catabolic pathways. Lumbar IVD herniation and instability due to IVD degeneration, lead to proliferation of bony and soft tissues, resulting in spinal stenosis and compression of the cauda equina, which can manifest in patients as low back pain, lameness, and neurological deficits.

The pathogenesis, biomechanics, clinical signs and therapy of caudal lumbar IVD herniation are similar between humans and dogs. Consequently, studies in humans with lumbar IVD herniation can contribute to a better understanding and treatment of this disease in dogs, and vice versa. Current treatments in both species aim at alleviating symptoms. In general, patients will be treated with conservative management such as physical therapy, weight reduction, structured exercise, and analgesics. If patients are refractory to conservative therapies, and/or neurological deficits progress, surgery is recommended. Surgical treatment consists of decompressive laminectomy with discectomy or sequestrectomy, possibly in combination with foraminotomy and/or facetectomy, to relieve compression on neural tissue. However, drawbacks of these methods include collapse of disc height, which may increase intervertebral instability and accelerate IVD degeneration causing re-herniation. In severe cases with instability of the lumbosacral (LS) junction, fixation and fusion of the affected segment, or replacement of the IVD with an endoprosthesis can be performed. In human medicine, interspinous spacer devices have also been used to treat lumbar stenosis in a minimally invasive way. By distracting the spinous processes and/or limiting extension of the lumbar spine, the spinal canal area, as well as the width and area of the intervertebral foramen, are preserved and increased. However, controversy remains whether interspinous spacers have better or worse outcomes than regular decompressive surgery, as they have been associated with a high rate of complications, higher costs, and a higher incidence of reoperation. Nevertheless, none of the aforementioned treatments restore the functional integrity of the human nor the canine IVD.

A relatively new approach to cartilage regeneration that originates from the field of osteoarthritis, is temporary joint distraction. Clinical improvement after temporary knee and ankle joint distraction was maintained for at least 2 and 7 years, respectively. Although the exact underlying mechanism is not known yet, this surgical technique is based on the hypothesis that osteoarthritic cartilage has some regenerative
capacity when it is mechanically unloaded by temporary distraction. Because of the similarities between articular cartilage and the IVD, several experimental studies have focussed on segmental distraction of the IVD to provide conditions for biological attempts of regeneration.28, 29

Distraction of both the IVD and facet joints can be achieved by using a pedicle screw-rod fixation (PSRF) device. This type of device, whether or not in combination with an intervertebral cage, has been used extensively in human medicine, and to a much lesser extent in veterinary medicine, in order to provide biomechanical stability, to decompress neural elements, to correct deformities, and to allow for spinal fusion.30 Nevertheless, fixation of a spinal segment alters the biomechanics of the spinal column, and it is assumed that the loss of motion at the fused level results in an increase of motion and load at the adjacent, unfused segments.31-33 Placing a fixation and distraction device temporarily, could allow biological repair of the IVD, but might prevent changes in adjacent segments. In human cadavers lumbar distraction predictably reduced pressure in the NP.34 In several studies in rabbits using a compression model to create IVD degeneration, distraction of the IVD showed signs of tissue repair at a biological, cellular, and biomechanical level.35-37 To our knowledge, the effect of temporary IVD distraction in animals with spontaneous IVD degeneration has not been evaluated before. Therefore, in this study, we assessed the safety and efficiency of temporary distraction in a dog with spontaneous IVD degeneration and clinical signs. As the dog is a suitable animal model for IVD degeneration in humans, this case report could provide valuable translational information.38, 39

Clinical and orthopedic examination
A 5-year-old, 31 kg, intact male Greyhound was presented to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht because of low back pain. The dog was owned by the Faculty of Veterinary Medicine and used as a blood donor and for teaching purposes. All procedures were approved and conducted in accordance with the guidelines set by the Animal Experiments Committee (DEC) of Utrecht University (experimental number: 2012.III.03.029), as required by Dutch regulation.

On clinical examination the dog showed a slight kyphosis, a shortened and stiff stride of the pelvic limbs, pain on palpation of the LS junction, and a painful response to the LS extension test. The dog showed no neurological deficits. Survey lateral and ventrodorsal radiographs of the LS area were obtained under sedation (t₁) and showed minimal mineralization of the L6 – L7 IVD. MR images of the LS area were obtained under general anesthesia (t₁) using a 1.5 Tesla scanner and a Sense NeuroVascular 16 top-off coil (Phillips Healthcare, Best, The Netherlands). T1-weighted, T2-weighted and T2-mapping
sequences were acquired in the sagittal and transverse planes according to the protocol previously described. On the sagittal planes a mild protrusion at L7 – S1 was seen, and slight degeneration of the L4 – L5 and L7 – S1 IVDs was noted. The dog was treated orally with a nonsteroidal anti-inflammatory drug (carprofen) in a dosage of 2 mg/kg, twice daily. As the dog showed no improvement on medical treatment, temporary distraction of the LS segment was performed (t0).

**Temporary distraction of the LS junction**

*Placement of the PSRF and distraction*

Temporary distraction of the LS junction by pedicle screw-rod fixation under general anesthesia was applied by an experienced veterinary surgeon (BPM) and orthopedic surgeon (FCÖ). The surgical procedure and insertion of the pedicle screws is described in detail by Smolders et al. and Meij et al., respectively. Briefly, a dorsal median incision was made, extending from the spinal process of L6 to S1. L7 and S1 were identified, and an awl was used to prepare the pedicle screw entry holes. Four 25-mm long, 4-mm wide titanium pedicle screws (USS Small Stature, Synthes®, Zeist, The Netherlands) were inserted into the pedicles and vertebral bodies of L7 (2) and S1 (2). Placement of the pedicle screws was checked under fluoroscopy. Two 5-cm long, 6-mm wide titanium rods were used to connect the L7 pedicle screws with the two ipsilateral S1 pedicle screws. The rod was slightly adjusted with a rod bender to acquire optimal alignment with both the screw heads. Prior to tightening of the sleeves and nuts on the screw heads, 5 mm distraction was applied with a Gelpi distractor to the pedicle screws over the L7 – S1 junction. A part of the cauda equina was exposed due to partial rupture of the ligamentum flavum, hence a splash block of morphine (0.1 mg/kg in 2 ml of 0.9% NaCl) was given and a small epidural autologous free fat graft was placed.

*Removal of the PSRF*

Distraction was applied for 3 months, based on joint distraction studies in humans with severe osteoarthritis of the ankle. After 3 months the PSRF was removed in a second surgery (t3). A dorsal median incision was made, extending from L7 to S1. The distraction device was visualized and nuts and sleeves were carefully untightened and the rods were removed. During removal of the 4 pedicle screws, it was noticed that the L7 pedicle screws were more firmly seated in the bone than the S1 pedicle screws. A bacteriology swab was obtained from one of the screw holes. Wounds were closed in a standard fashion.

*Follow up*

Radiography, and force plate analysis were performed before surgery (t-1), at placement of the distraction device (t0), after 3 months of distraction at removal of the device (t3), and again at 6 (t6) and 9 months (t9) follow-up. MR images were obtained at t-1, t3 and t9 (Table
1). Disc height index (DHI) was calculated for L7 - S1 at all time points on radiographs, according to the method described by Masuda et al.\textsuperscript{43} The surface area of the IVD was measured at all time points on lateral radiographs using Adobe Photoshop CS6 (Adobe Systems, San Jose, USA). Prior to measuring the surface area, radiographs at all time points were magnified in the same degree, thereby using the length of the vertebral body L7 as a reference. Mid-sagittal slices of T2-weighted images were used to evaluate the grade of degeneration at all time points. IVDs were evaluated according to the Pfirrmann classification validated for dogs by Bergknut et al.\textsuperscript{44} T2 values were calculated and analyzed according to a previously described method by our group.\textsuperscript{40} Force plate analysis as previously described by Suwankong et al.,\textsuperscript{45} was performed to measure ground reaction forces (peak vertical force \(F_z^+\)), peak braking force \(F_y^+\), and peak propulsive force \(F_y^-\)) as objective outcome parameters to assess the efficacy of treatment.

<table>
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<th>Table 1. Follow up schedule.</th>
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<td>Time (t\textsubscript{month})</td>
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<td>Preoperatively</td>
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<td>At placement of distraction device</td>
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<td>At removal after 3 months of distraction</td>
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<td>3 months</td>
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### Results

**Surgical management**

*Placement of pedicle screw-rod fixation and distraction*

After placement of the PSRF device, the dog was admitted to the surgical ward and treated intravenously with fentanyl (4 \(\mu\)g/kg/hr), ketamine (4 \(\mu\)g/kg/min), carprofen (4 mg/kg once daily), and amoxicillin/clavulanic acid (20 mg/kg three times a day). The fentanyl/ketamine was tapered down the next day, and intravenous methadone (0.2 mg/kg) was given once, before oral tramadol (3 mg/kg three times a day) was started. The dog was evaluated daily by means of a general and orthopedic examination performed by a veterinarian (NW), and pain was assessed according to the short form of the Glasgow composite pain scale.\textsuperscript{46} One day after surgery, swelling in the back of both knees was noticed. Based on ultrasound findings, cytology and bloodwork (hematology), this swelling was most likely associated with congestion or local bleeding. Five days after surgery, tremors of the head were noticed. Tramadol was stopped for 24 hours and bloodwork was performed; biochemistry and hematology showed no abnormalities, and no difference was noticed without providing tramadol. These tremors spontaneously resolved after 7 days. Four weeks after insertion of the PSRF + distraction device, the dog showed mild...
kyphosis and stiffness of the lower back. Radiographs of the LS area showed no abnormalities, and a stable presentation of the pedicle screws and rods was noted. The dog was treated with carprofen (4 mg/kg once daily) for 14 days. Thereafter the pain medication was discontinued. The dog’s activity was restricted for 6 weeks, and was only allowed to go on short leash walks.

**Removal of pedicle screw-rod fixation**

After removal of the PSRF device the dog was admitted to the surgical ward and was treated intravenously with dexmedetomidine (1 μg/kg/hr) for 24 hours, buprenorphine (20 μg/kg four times a day) for 2 days, and orally with carprofen (4 mg/kg once daily) for 10 days. After the buprenorphine was stopped, tramadol (3 mg/kg three times a day) was started and given orally for 14 days. Again, the dog’s activity was restricted for 6 weeks, and was only allowed to go on short leash walks. Recovery of the dog was uneventful. The bacteriology swab taken from a pedicle screw hole at removal of the device tested negative.

**Radiographs**

Despite the minimal mineralization of the L6 – L7 IVD, radiographs were unremarkable. In none of the radiographs that were obtained during or after distraction, implant failure was seen. DHI in L7-S1 remained unchanged after distraction (t₁ versus t₀)(Figure 1). At 3 months of distraction, immediately after removal of the device (t₃), and at 6 months (t₆), DHI decreased with 8%. At 9 months (t₉) the DHI returned to the initial value (t₀). Although the DHI was not different before and after distraction (t₁ vs t₀), assessment of the complete IVD revealed distraction of the dorsal part of the IVD, whereas compression of the ventral part (Figure 1). Indeed, the IVD surface area on lateral radiographs increased by 15% and 20% at t₀ and t₃, respectively, compared with t₁. At 6 (t₆) and 9 (t₉) months, the IVD surface area decreased to values slightly higher, i.e. 1% and 2%, respectively, than the initial value at t₁.

**MR imaging**

At 9 months, i.e. 6 months after removing the distraction device, fibrous tissue with a low signal intensity on both T1-weighted and T2-weighted images surrounding both facet joints of L7-S1 was noticed. Furthermore, tissue with a high signal intensity on T1-weighted images and a low signal intensity on T2-weighted images, consistent with either fibrous or fat tissue, surrounded the facet joints of L6-L7. Pfirrmann scores on T2-weighted MR images in the L7-S1 remained grade II at all time points. T2 mapping value was highest (208.7 ± 46.3) directly after removal of the distraction device, but decreased to a value lower than pre-operatively (165.9 ± 46.4) and at 6 months after removal (148.8 ± 58.9)(Figure 1).
Figure 1. Lateral radiographs, and lateral T2-weighted magnetic resonance (MR) images of the lumbosacral junction of a dog with degenerative lumbosacral stenosis exhibiting low back pain and concurrent mild degeneration of the L7-S1 intervertebral disc (IVD). Temporary distraction of the lumbosacral junction was applied with the aid of pedicle screw-rod fixation. The dog was evaluated at the following time points: before (t1), directly after application of the distraction device (t0), after 3 months of distraction at removal of the device (t3), and again at 6 (t6) and 9 months (t9) follow-up. Disc height index (DHI) and area of the IVD were measured on lateral radiographs, with t1 set at 100%. T2 values are mean T2 mapping values (± SD) in the NP, obtained at the same time as the T2-weighted images. NP: not performed.
**Force plate analysis**

Before distraction the pelvic/thoracic (P/T) peak vertical force (Fz+) and P/T peak breaking force (Fy') were slightly higher than reference values in control animals, whereas the P/T peak propulsive force (Fy-) ratio was comparable with that in dogs with DLSS as described by Suwankong et al. (Figure 2). A decrease of 16% of the P/T Fy' was noticed after three months of distraction. The P/T Fy' slowly increased after the distraction device was removed and resulted in a value at 9 months follow up that was 4% higher than the initial preoperative P/T Fy' value.

![Figure 2](image)

**Discussion**

In a dog with clinical signs due to DLSS with early IVD degeneration we demonstrated safe temporary fixation and distraction of the LS IVD and facet joints by using PSRF. Permanent instrumented fixation of the LS junction has gained more interest in veterinary literature, and most researchers focused on achieving permanent and optimal stabilization of the spinal segment by using PSRF. Temporary fixation and distraction of the IVD however, to unload and possibly allow repair of the IVD, to our knowledge has not been investigated in humans and dogs with clinical signs of degenerative disc disease in the lumbar region before. In the present study signal intensity was compatible with Pfirrmann grade II degeneration on T2-weighted MR images pre-operatively. During and after temporary static distraction the signal intensity on T2-weighted images remained unchanged. Furthermore, the T2 value, a quantitative MR imaging technique shown to be more sensitive in detecting qualitative changes over the course of IVD degeneration, was highest directly after removal of the distraction device, which may indicate an increase in water content. However, at 9 months follow-up the T2 values decreased by 10% compared with the pre-operative value. Despite the initial increase in the T2 value, DHI
slightly decreased (8%) after removing the distraction device, but disc height recovered to the pre-operative value at 9 months follow-up. Nevertheless, precision of these results could not be indicated, as statistical analysis was not performed in this one dog. Even more so, in a previous study in Beagle dogs, a 21% variability was seen in T2 maps, which may indicate that the T2 mapping values reflect a physiological variance.

The fibrotic changes of the facet joints at both levels may be caused either by immobilization of these joints during the distraction period and/or by an increase in biomechanical loading after removal of the static unloading. Shortening of the distraction period to 4 or 6 weeks, might reduce formation of fibrosis and most likely has similar regenerative effects, as biomarker-turnover of cartilage and bone tissue increase within the first four weeks of joint distraction, and thereafter stabilize. Whether the fibrotic changes are a consequence of duration and/or placement of the pedicle screws, distraction itself, and have a negative effect on outcome parameters, should be evaluated in a larger group of patients, and over a longer period of time. As depicted in Figure 1, the effect of distraction in each of the separate IVD compartments may well be different, as the dorsal part seems more distracted, while the ventral part seems slightly compressed.

The current *in vivo* study set up in this study, did not allow for histopathological, biochemical and/or biomolecular evaluation of IVD tissue. In a rabbit IVD compression model resulting in a decreased signal intensity of the IVD on MRI, temporary dynamic distraction and unloading of a spinal segment by using flexible spring mechanisms showed reestablishment of the physiologic signal intensity on MRI. Contrasting findings between the dog with spontaneous IVD degeneration and the rabbit model with induced IVD degeneration were observed. Several aspects can clarify this discrepancy. First, the type of distraction device differed in both animal studies, i.e. static in the dog, versus dynamic in the rabbit model. Dynamic loading of a specific magnitude, frequency and duration has been shown to maintain the balance between anabolic and catabolic pathways within the ECM. Variations in loading have been associated with enhanced nutrient transportation in and out of the IVD. In a more static loading condition, decreased nutrient supply might have limited ECM synthesis, resulting in a lower expression of water-binding proteins, and a consequently lower signal intensity on MRI. Furthermore, pins in the rabbit were placed perpendicular to the spinal segments. Pedicle screws in the dog could not be placed strictly perpendicular, due to anatomical limitations. As the screws could only be placed under a slight angle, distraction of the dorsal, but compression of the ventral part of the joint was applied. Finally, differences in genetic background, and/or to the stage of IVD degeneration may have resulted in a difference in the degenerative as well as the regenerative processes within the spontaneously and induced degenerated IVDs.
Force plate analysis can be used to objectively and accurately assess gait in dogs. Dogs with DLSS have been shown to have decreased propulsive forces of the hind limbs (P/T Fy ratio). The initial decrease of the P/T Fy in this dog during PSRF + distraction is in line with findings in literature, and is most likely associated with a reduction in pelvic limb muscle strength and volume within the rehabilitation period. In previous studies, the P/T Fy ratio initially decreased after fixation of the LS joint by using PSRF at 6 and 12 weeks, but increased at 6 months after surgery. Even more so, in the most recent study, a limited number of dogs showed P/T Fy ratios more than three years after surgery that were comparable with ratios in normal dogs. Interestingly, in the present study the propulsive forces of the dog also improved 9 months after removal of the PSRF + distraction device. Shortening of the distraction period might decrease post-treatment stiffness, and may reduce this initial decrease in P/T Fy, and/or accelerate improvement. A longer follow-up time is needed to give more insight into the long term outcome.

In this study, we used a commercially available PSRF device to provide distraction of the LS joint, that is designed to stabilize and fixate a spinal segment permanently. One of the limitations of using such a device is that the amount of distraction cannot be quantified. Furthermore, surgical intervention would ideally consist of one procedure to place the distraction device, and a second minimally invasive procedure to remove it. As this device is designed to be placed permanently, removal via a minimally invasive procedure is limited. Distraction of knee joints in dogs, and IVDs in rabbits, have been performed using external fixateurs, that are tolerated well by the animals. In this procedure an external device, including a calibrated spring, serves as a distractor and is attached to bone pins that are placed on either side of the joint under fluoroscopic guidance. By using a spring, a constant controllable dynamic decompression over the entire unloading time can be established. After the distraction period, spring and pins can be removed via a minimal invasive surgical procedure. However, as the distance of this device to the spine is greater than in an accessible joint like the knee or the ankle, creating a similar precise leverage effect at the spine level to apply an equal amount of distraction in all segments of the IVD, comparable to that in a joint, seems quite challenging. Currently, none of the aforementioned devices are commercially available, and need to be custom-made.

Altogether, temporary PSRF in combination with distraction in this patient has been shown to be safe, to improve clinical signs and to retain IVD height at 9 months follow-up. In order to evaluate the safety and clinical efficacy of this technique in the treatment of DLSS and regeneration of the IVD, more patients need to be included in future studies, and longer follow-up times need to be considered.
Acknowledgements

The financial contribution of the Dutch Arthritis Foundation (LLP22) and donation of the pedicle screws and rods by DePuy Synthes are gratefully acknowledged. We would like to acknowledge Arie Doornenbal for his technical assistance, Greet Bouwman and Rob Sap for their help in obtaining MR images, and Danny Tsui for his help in analysis of T2 maps.

References


Chapter 8
General discussion
General discussion
A better understanding of the etiopathology of IVD degeneration in man and dog, together with the current clinical therapeutic limitations, have provided the basis to investigate novel regenerative therapies in this thesis that could benefit both species. Bioactive substances were delivered intradiscally, either alone, or in combination with injectable sustained release systems, in a canine model predisposed to spontaneous IVD degeneration. Furthermore, PSRF with and without distraction of the lumbosacral junction was investigated in canine patients. Results, challenges, and future perspectives are discussed below.

Intradiscal injection
Intradiscal delivery of biomaterials, exogenous growth factors, genes, and cells, either alone or in combination, are promising regenerative approaches for intervertebral disc (IVD) repair. Although intradiscal injection via a minimally invasive procedure seems an attractive technique, it should be considered with care. In vivo and in vitro studies have shown that the ratio of needle diameter to disc height, and the ratio of injection volume to total nucleus pulposus (NP) volume should not exceed 40% and 66%, respectively, otherwise it may, in itself, induce IVD degeneration.1-5 Clinical discography in human patients, even when using small diameter needles (22 – 25G) and controlled pressurization, has been associated with accelerated degeneration, development of disc herniation, greater loss of disc height, greater loss of water signal on T2-weighted magnetic resonance imaging (MRI), and the development of reactive endplate changes compared with matched-controls over 10-year follow-up.6 However, the injection material itself might also have been (partially) responsible for the induction of IVD degeneration, as a recent study has shown that contrast media used in discography also has a direct, dose-dependent cytotoxic effect on human IVD cells in vitro.7 Hence, these studies indicate that caution is warranted for the use of discography, but they do not necessarily indicate that regenerative treatment strategies based on local delivery also pose a risk for the IVD itself.

Regenerative therapies may be applied safely in human and veterinary patients, when cautiously selected injection volumes, materials, and needle diameters, based on aforementioned studies, are used. In order to investigate the long-term effects of intradiscal injection of a sham condition in a representative spontaneous animal model, i.e. chondrodystrophic (CD) dogs, in vivo and post mortem, we set up a study in laboratory beagle dogs (chapter 2). Ratio of needle diameter to disc height and ratio of injection volume to NP volume were 11% and 20%, respectively, and thus way below the critical values. We showed that a volume of 40 μl of this condition through a 27G needle induced no further degenerative changes in these early degenerated IVDs over a course of 6 months. At the level of gene expression we found that sham-injected IVDs showed
increased caveolin-1 (CAV-1) gene expression compared with non-injected IVDs at 6 months follow up. Decreased CAV-1 has been associated with early IVD degeneration in CD dogs and with senescence of NP cells. However, the biological relevance of this finding in our study can be questioned, as although relative CAV-1 expression was approximately two-fold upregulated, the protein expression of caveolin-1 within the NP or the annulus fibrosus (AF) was not detected, and all other analyses (biomolecular analyses of genes related to apoptosis, and biochemical, and histopathological analyses) showed that IVD integrity was not affected in sham-injected IVDs.

Intradiscal application

Growth factors

A number of studies have investigated the potential regenerative role of bone morphogenetic proteins (BMPs) in degenerative IVD disease. rhBMP-7 appeared to be a promising candidate for IVD regeneration, as it has been shown to stimulate extracellular matrix (ECM) production of rabbit, bovine, and human IVD cells in vitro, and when applied intradiscally in vivo, to induce regenerative effects in a rabbit model of induced IVD degeneration. In line with literature, we have shown in chapter 3 that rhBMP-7 also stimulated glycosaminoglycan (GAG)-rich matrix production of NP cells of CD dogs in vitro. However, in vivo studies in a spontaneous CD dog model (chapter 3), showed extensive extradiscal bone formation after intradiscal application of rhBMP-7 dosages of 25 and 250 μg, without any clear regenerative effects on the IVD. Differences in outcome between CD dogs and rabbits in vivo could be associated with the type of animal model. Cell type variation related to differences in genetic background may play an important role, with a concomitant difference in degenerative, as well as regenerative pathways. The presence of notochordal cells (NCs) for example, differs between different species and has been suggested to be favorable in promoting regeneration and repair. Furthermore, pathological pathways might considerably differ between induced and spontaneous IVD degeneration models and accordingly involve different cell types and cellular responses within the IVD.

However, early degenerated canine NP cells responded in a similar way to rhBMP-7 as rabbit NP cells in vitro, indicating a difference in rhBMP-7 activity in vitro and in vivo in the canine model. An explanation for the differences between the animal models, as well as the differences between in vitro and in vivo studies in the canine model, could be found in the complex BMP signaling pathway in vivo. The effect of rhBMP-7 is mediated via specific BMP receptors that activate the intracellular signaling protein SMAD1/5/8. While using the same canonical SMAD pathway, BMPs can have diverse effects. Nuclear cofactors interact with the SMADs in regulating particular target genes, depending on the cellular context. Several factors that regulate members of the BMP signaling pathway are
identified at the cell surface, in the cytoplasm, and in the nucleus. Many of these factors are BMP-inducible and inhibit the BMP pathway, creating a negative feedback loop. Even more so, members of the BMP-SMAD pathway can also cooperate with components of other signaling pathways. In addition, extracellularly, the activity of BMP-7 can be regulated by natural BMP antagonists, e.g. noggin, that block the binding sites of the BMP receptors. Relative gene expression of *NOG* was significantly upregulated in canine IVDs treated with 25 μg vs 2.5 μg and sham treated IVDs (chapter 3), suggesting that BMP antagonists may indeed play a role in the regulation of *in vivo* BMP-7 signaling. Determining relative gene expression of *NOG* in combination with protein levels and functional studies in NP cells of CD dogs and rabbits stimulated with rhBMP-7 *in vitro* as well as *in vivo*, would provide more information on its role in BMP-7 signaling *in vitro* and *in vivo*, and shed some light on possible differences between the induced and spontaneous animal model.

Another possible explanation for the inconsistency between *in vitro* and *in vivo* findings in the canine study could lie in the different frequency of rhBMP-7 administration: biweekly *in vitro* vs. a single dose *in vivo*. As most proteins have a short biological half-life and can diffuse out of tissues, the efficacy of the single dose *in vivo* could have been limited. Bioavailability of rhBMP-7 *in vivo* can be increased by using a sustained release system that allows controlled release of low dosages of rhBMP-7 over a prolonged period of time. Nevertheless, preserving growth factor bioactivity during incorporation into the biomaterial, and creating optimal physical and chemical properties of the biomaterial to effectively sequester the growth factor, are major challenges in engineering growth factor delivery systems. Interestingly, in a recent *in vivo* study in adult goats, BMP-2/7 heterodimers that were conjugated to a fibrin/hyaluronic acid (FB/HA) hydrogel, were intradiscally injected into chemically induced mildly degenerated IVDs. Sustained release of transglutaminase BMP-2/7 proteins was demonstrated, whereas no effects on IVD regeneration were shown. The authors suggested that the BMP dosages were too low, follow-up time was too short, cleavage of the transglutaminase-BMP complex from the hydrogel was ineffective, and/or release of the conjugated BMPs was insufficient. In future studies, these aspects, as well as the risks of extradiscal bone formation should be addressed.

Intradiscal injection of rhBMP-7 might have provided an osteo-inductive stimulus, either through suboptimal delivery of rhBMP-7 by the injection technique, or by diffusion out of the NP. The injection technique can be refined by using fluoroscopy or CT-guided imaging, allowing precise needle placement. Diffusion of a regenerative agent out of the IVD might be enhanced by biomechanical forces and/or disorganization of the lamellar structure of the AF that are part of the early IVD degeneration process. Leakage of a bioactive
substance out of the IVD after injection may be prevented by filling the injection tract in the AF inside-outwards with a biocompatible glue, that is delivered through an advanced smart double or triple chamber device.\textsuperscript{25, 26} Although closure and repair of the AF are important topics in current research on regeneration of the IVD, they were beyond the scope of this thesis.

**Biomaterials**

To minimize the number of intradiscal interventions, long term delivery of bioactive substances into the IVD can be achieved by using sustained release systems. Intradiscal delivery systems provide several other advantages, such as protection and improved biocompatibility of the loaded compound, and prevention of systemic side effects. An appropriate biomaterial should sufficiently prolong delivery of an active substance, or facilitate tissue repair, while degrading completely. Specifically for NP regeneration, limited amounts of these biomaterials are preferably injected through needles with small diameters. Within the Biomedical Materials IDiDAS project, biodegradable sustained release systems that could be injected through 26 – 29G needles, e.g. hydrogels and microspheres, were developed and tested \textit{in vitro}. These synthetic biomaterials could be reproducibly manufactured, while their mechanical and thermal properties could easily be tuned.

Newly developed biodegradable polyester amide polymer (microspheres) PEA(M)s (\textit{chapter 2}) and a poly-N-isopropylacrylamide pNIPAAM MgFe-layered double hydroxide (LDH) hydrogel (\textit{chapter 4}) have been shown to have a good biocompatibility \textit{in vivo} and could be safely injected through a 26G and a 29G needle, respectively. Implantation of PEAs intramuscularly, and the pNIPAAM MgFe-LDH hydrogel subcutaneously, induced a slight and moderate foreign body reaction, respectively. Although these reactions were not seen after implantation of these materials in the NP of the avascular IVD, aspecific cellular changes were noted in a limited number of IVDs. Future studies should provide more information on the origin of these reactions.\textsuperscript{27}

In order to use biomaterials as a future regenerative therapy, effectuating sustained release of bioactive substances in the degenerative IVD, their release profiles, and degradation in the intradiscal environment need to be explored. There are several challenges that still need to be addressed. Will loaded biomaterials remain at the place of injection, or will they be distributed throughout the IVD after intradiscal injection? Will they even be encountered in other organs outside the IVD? This will depend on the characteristics of the biomaterials themselves and the local environment. In order to obtain more insight into their \textit{in vivo} behavior, biomaterials could be labelled. Non-invasive real time \textit{in vivo} imaging of biomaterials can be achieved by CT detecting iodine-
containing moieties,\textsuperscript{28,29} by a Fluorescence – Assisted Resection and Exploration (FLARE\textsuperscript{TM}) surgical imaging system detecting invisible near-infrared (NIR) fluorophores,\textsuperscript{30} or by $^{19}$F-MRI detecting perfluorocarbon (PFC) nano-emulsions.\textsuperscript{31,32} Obviously, these labels should not interfere with degradation and release profiles of the biomaterials, bioactivity of the loaded substance, or the native IVD tissue. Although live imaging of labels in IVDs is successfully performed in rodent models,\textsuperscript{33} in large animal models this is still challenging, specifically in regions that are covered by large volumes of surrounding tissues, e.g. muscles, fat, and bone (vertebrae).

\textbf{Anti-inflammatory agents}

Sustained release systems have emerged as effective vehicles for local delivery of bioactive agents and they may play a prominent role in the relatively confined environment of the IVD. These systems enhance bioavailability of therapeutic agents over a time course necessary to achieve a certain cellular response, reduce the administration rate, and decrease systemic side effects. Several preclinical studies in induced IVD degeneration models in rats and rabbits have shown promising data with a limited number of different biomaterials, loaded with different therapeutic agents.\textsuperscript{34-38} As mentioned before, in contrast to animal models with induced IVD degeneration, studies in models with spontaneous IVD degeneration, targeting the underlying pathological processes that match human IVD degeneration, provide valuable translational information.

Targeting inflammation is one of the emerging treatment strategies of IVD degeneration and chronic low back pain. In \textit{chapter 4} we evaluated injectable pNIPAAM MgFe-LDH sustained release systems (hydrogels) loaded with a COX-2 inhibitor, celecoxib (CXB), in order to reduce intradiscal prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) levels over time in a dog model with spontaneous IVD degeneration. \textit{In vitro}, the CXB-loaded gels suppressed TNF-\textgreek{a}-induced PGE\textsubscript{2} production for a prolonged period, in contrast to a short-lived effect of a bolus of CXB. \textit{In vivo}, the pNIPAAM MgFe-LDH hydrogels loaded with CXB over a wide dose range, were shown to be biocompatible and to be safely injected in the IVD, without affecting the condition of the IVD. Despite suppression of PGE\textsubscript{2} levels by CXB-loaded pNIPAAM MgFe-LDH hydrogels \textit{in vitro}, sustained release of different loading dosages of CXB \textit{in vivo} only showed a nonsignificant decrease in PGE\textsubscript{2} levels over a period of 28 days. As CXB tissue levels \textit{in vivo} could not be determined, the release profile of the CXB-loaded sustained release systems \textit{in vivo} remained unclear. Differences in release profiles due to different pharmacokinetics of a bioactive agent \textit{in vitro} and \textit{in vivo} is a challenge in the field of sustained release.\textsuperscript{28,39} Although several parameters, such as pH, medium composition, and osmotic pressure can be mimicked \textit{in vitro}, the presence of different types of cells, biochemical mediators and/or other macromolecules, and biomechanical loading, can
influence release kinetics in vivo. To evaluate in vivo release profiles, one would ideally label sustained release systems with a marker that correlates with the release profile of the loaded substance, which can be quantified with MR or NIR imaging for example. Although several innovative techniques have been developed to label and track loaded substances in vivo, the field is challenged by limitations regarding specificity of the markers, and imaging in in vivo models larger than rodents.

Based on low percentages of COX-2 positive cells at immunohistopathology, we consider it very likely that the lack of PGE2 inhibition by the CXB-loaded pNIPAAM MgFe-LDH hydrogel was attributed to the limited presence of inflammation-induced COX-2. Although MR images, macroscopic and histologic parameters corroborated with a degenerative status of the IVD, this early phase of spontaneous IVD degeneration, without clinical signs, seemed not to be associated with increased PGE2 levels.

**Targeting inflammation in IVD degeneration**

The results of the sustained release of CXB in the canine spontaneous IVD degeneration model raised the important question if increased levels of PGE2 might only become evident or relevant in moderate and late IVD degeneration, when clinical signs are also present. Therefore, in chapter 5 we compared IVD samples collected from laboratory dogs without clinical signs to samples from companion dogs with clinical signs of IVD degeneration (IVD disease) that were treated surgically. The group of laboratory dogs as well as the group of surgically treated dogs consisted of CD and non-chondrodystrophic (NCD) dog breeds.

Classification of these samples by degeneration grade, without taking herniation into account, showed significantly higher PGE2 levels in degenerated IVDs compared with non-degenerated (healthy) IVDs, suggestive of an inflammatory component in IVD degeneration. These findings were supported by COX-2 protein expression in the NP and AF and numbers of macrophages, which both increased with degeneration grade. In order to evaluate the effect of degeneration grade in conjunction with herniation on PGE2, far more samples from CD and NCD dogs with similar herniation types, but with different degeneration grades should be compared, or vice versa. Within our study, we were only able to perform this analysis in a limited number of grade II IVDs from CD dogs presenting with different herniation types, showing increased PGE2 levels in protruded NP samples compared with NP in situ samples. As mentioned before, NCD dogs are predisposed to protrusion of the NP (Hansen type II) of degenerated lumbosacral and caudal cervical IVDs, and NP extrusion of degenerated thoracolumbar IVDs, while CD dogs are predisposed to explosive extrusion of the NP (Hansen type I) of degenerated cervical and thoracolumbar IVDs.
When using a classification scheme based on herniation type, elevated PGE$_2$ levels in IVDs from NCD dogs were associated with protruded and extruded samples, which was only the case in protruded NP samples in grade II IVDs in CD dogs. Findings in extruded tissues were in line with a recent *in vitro* study, which showed that swelling of NP tissue in bovine NP explants, i.e. extruded NP tissue, triggered PGE$_2$ production. However, in that study elevated PGE$_2$ levels were measured in medium, and not in NP tissue itself.$^{45}$ Within our study design it was not possible to include surrounding tissues, such as ligaments, the spinal cord and the epidural space in our study. Hence, we could not rule out that PGE$_2$ and cytokine levels were influenced by diffusion to, or infiltration from the epidural space. Determining PGE$_2$ and cytokine levels in these surrounding tissues in future studies may shed some light on the general aspects of the inflammatory response. Histological findings described in studies on canine herniated IVDs showed an acute inflammatory reaction in extruded IVDs, characterized by neutrophils and macrophages, while in protruded IVDs a more chronic inflammatory response was shown, characterized by macrophages, lymphocytes and plasma cells.$^{27, 46}$ To clarify whether PGE$_2$ is produced by resident IVD cells, or by attracted inflammatory cells, surgical IVD samples from CD and NCD dogs should not only be examined for PGE$_2$ content, but should also be analyzed by histopathology at the same time.

Currently, it remains unclear whether elevated PGE$_2$ levels and COX-2 expression in degenerated IVDs represent a physiological inflammatory response to promote tissue repair, or that they reflect an excessive inflammatory response with detrimental effects on tissue integrity, that contributes to the pathogenesis of IVD degeneration and/or disease. The response in CD dogs is likely to be different from the response in NCD dogs. Inflammatory cytokines have been shown to stimulate IVD cells to produce chemotactic factors, which attract macrophages, neutrophils and T cells.$^{47}$ This process corresponds well with the typical clinical presentation of NCD dogs, which develop protrusion of the AF (type II herniation) over time, associated with pain. However, the clinical cascade of IVD disease in CD dogs might differ, as these dogs show acute signs, associated with NP extrusion (type I herniation) and focal stimulation of the spinal cord meninges with concurrent pain. From a clinical point of view, decompression surgery is recommended if dogs present with clinical signs, and diagnostic work-up indicates that extruded material causes compression of neural tissue. Thus, targeting PGE$_2$ levels by intradiscal delivery of sustained release systems loaded with an NSAID to provide effective analgesia, seem most beneficial in NCD dogs that present with low back pain, due to protrusion of the IVD. Therefore, intradiscal delivery of a sustained release system loaded with an NSAID in dogs with moderate degenerative lumbosacral stenosis (DLSS) due to IVD degeneration and herniation is currently focus of an ongoing research study.
Whether PGE2 is a harmful factor under all circumstances remains a matter of debate. PGE2 has been shown to have anabolic and anti-catabolic effects on ECM in the IVD, which may be concentration-dependent.\textsuperscript{48-52} Low concentrations of PGE2 have been described to stimulate proteoglycan synthesis in rat chondrocytes, whereas higher doses have been described to decrease proteoglycan synthesis in rat NP cells.\textsuperscript{50, 51} Based on the nonsignificant decrease in PGE2 by the CXB-loaded pNIPAAM MgFe-LDH hydrogels in CD dogs without clinical signs, baseline levels of PGE2 are most likely established by COX-1. During inflammation, administration of COX-2 inhibitors will ensure retention of these basal levels, whereas detrimental effects of inflammatory levels of PGE2 can be prevented.

Within veterinary practice, NCD dogs with low back pain that do not respond to systemic NSAIDs are common, and mild or severe side effects are often presented. An ongoing preclinical study at the University Clinic for Companion Animals is evaluating the effect of intradiscal delivery of a sustained release platform loaded with CXB in NCD dogs with low back pain. This study may improve veterinary care, but may also provide preclinical information on possible translation towards human medicine. Although this treatment seems promising, quite some hurdles are ahead. First of all, will CXB intradiscally provide sufficient analgesia? Will the effect be as long as the entire period of sustained release of CXB or does the effect disappear below a certain threshold level? Does the injection need to be repeated, which interval should be used, and what will be the long term effects of (multiple) injections in the degenerated IVD?

Stabilization and unloading of the degenerative IVD
Surgical management is the treatment of choice for dogs and humans with refractory symptoms of disc degeneration and herniation leading to lumbar stenosis. In humans, the benefits of spinal fusion subsequent to decompression are generally well accepted. Nevertheless, in selected patients (non-instrumented) decompression without fusion can be sufficient, thereby avoiding complications associated with instrumentation and fusion, e.g. increased surgical time and costs, neural injury, and additional bloodloss.\textsuperscript{53} Fixation and interbody fusion have been shown to restore and maintain disc height, and increase stability of the treated spinal segment.\textsuperscript{54} Although the safety and efficacy of pedicle screw-rod fixation (PSRF) for the treatment of DLSS in large breed dogs has been established, long-term results were not reported thus far.\textsuperscript{55, 56} In \textit{chapter 6} we showed that dogs treated with PSRF because of severe DLSS disease, in which no other treatment was available, had a good clinical outcome at long-term follow up. These patients were referred to our University Hospital for Companion Animals because of the complexity of a surgery, failure from a previous surgery, and in a minority of cases even for a treatment of last resort. Interestingly, clinical remission and recurrence rates in these complicated patients were similar to those reported for decompression by dorsal laminectomy alone in
uncomplicated cases. Even when combined with autologous bone grafts, showed no interbody vertebral bone fusion of the LS junction on the long term most probably due to insufficient abrasion of the endplates. However, no significant difference in outcome in human and canine patients was shown between patients with successful spinal fusion and patients that failed to develop interbody vertebral fusion after spinal fusion surgery. Thus, as solid interbody vertebral bone fusion is not necessarily associated with decreased back pain, it can be questioned if interbody bone fusion in canine patients should be the ultimate goal in stabilization with PSRF.

The rigid PSRF construct is in principle designed to provide stability to a spinal segment. In chapter 7 we have used this type of construct in combination with temporary distraction in a canine patient with early clinical signs of DLSS, in order to regenerate and repair the lumbosacral IVD. Joint distraction has been shown to be successful in postponing ankle arthrodesis and knee arthroplasty in end stage osteoarthritis patients, although regeneration of truly hyaline articular cartilage was not unequivocally demonstrated. In several studies in rabbits using a compression model to create IVD degeneration, distraction of the IVD showed signs of tissue repair at a biological, cellular, and biomechanical level. Temporary distraction was shown to be a safe procedure in the canine patient, however, in contrast to findings in induced rabbit IVD models, no clear signs of regeneration were seen on radiographs and/or MR images at 9-months follow up. The exact mechanism how joint distraction initiates structural tissue repair is still unknown, but seems to be driven by biomechanical effects; cell signalling pathways have been identified that mediate cellular and transcriptional responses to (un)loading of articular cartilage. Furthermore, similar to the effects of negative pressure on skin wound repair, negative pressure created in a distracted joint might also accelerate cartilage repair. Future studies in a larger cohort of patients should elucidate whether temporary dynamic or static distraction is needed to establish IVD repair. Unfortunately, recruitment of patients for this distraction study was challenging, as dog owners were reluctant to have a second, quite invasive, surgery performed in their dog. A distraction device, possibly equipped with a calibrated spring, that could be placed and removed via a minimally invasive procedure could overcome this issue. Currently no such device is commercially available, however, promising results in future might encourage manufacturers to develop commercially available distraction devices that can be easily applied in canine, or even human patients.

**Considerations and perspectives for future treatment strategies**

Several innovative therapies to treat and/or reverse IVD degeneration are being investigated, including intradiscal delivery of various compounds, e.g., anti-inflammatory drugs, growth factors. This approach is expected to play a key role in future
treatment strategies for IVD regeneration. Biomaterials will be pivotal here, as they can serve as cell, drug, and/or growth factor carriers, and they can have the ability to attract native cells, to protect the loaded substances from the hostile degenerated IVD environment, and to provide a three-dimensional environment for regeneration.\textsuperscript{70, 71}

When applied intradiscally, biomaterials should possess many different properties including the following: (a) maintain activity of the loaded substance, (b) provide anchorage to the extracellular matrix, in situ formation, in order to prevent leakage of the injected gel and its loaded substance, (c) withstand biomechanical forces and provide a permissive environment for loaded cells and/or substances, (d) maintain steady degradation and release profiles, and (e) produce degradation products that are not toxic to the environment. Determining intradiscal release profiles and/or distribution of loaded substances \textit{in vivo} would make an important contribution to adjust loading doses. Evaluation of efficacy using sophisticated imaging techniques, e.g. \textsuperscript{19}F MRI, and T1ρ and T2-mapping, that allow noninvasive detection of the injected compounds and identification of subtle changes within the ECM, respectively,\textsuperscript{72, 73} can be used to relate release profiles to biological effects, thus allowing for optimization towards maximum efficiency.

Loading delivery systems with steroidal or non-steroidal anti-inflammatory drugs that specifically target inflammation seem to be a logical option. Although the use of steroids as an intradiscal anti-inflammatory therapy is a matter of debate,\textsuperscript{74, 75} it has been shown to be effective at short-term follow-up in a subset of human patients with low back pain.\textsuperscript{76, 77} High doses of steroids have been shown to slow down the natural resorption of extruded NP tissue in a rabbit tissue model.\textsuperscript{78} Specific targeting of the eicosanoid pathway by intradiscal injection of a selective NSAIDs may be a more suitable and safe approach. Long-term follow up should also provide more information on the effects of an anti-inflammatory treatment on IVD matrix integrity. As IVD disease in dogs and humans share many similarities, the results of the currently ongoing clinical trial in NCD dogs with low back pain due to DLSS can provide important information for translation into an effective therapy for humans. Even if this specific treatment would only provide long-term analgesia, it could still be a valuable treatment option for dogs with DLSS, whose owners decline surgical treatment, e.g. for financial reasons. In fact, in specific situations in humans, this kind of treatment might ethically be preferred over currently available treatments, e.g. because of considerable comorbidity in elderly people treated surgically.

Rather than alleviating signs associated with IVD degeneration, such as pain and inflammation, regenerating the IVD would be preferable. Unfortunately, to date there is no adequate regenerative therapy available. The optimal strategy will depend on several factors, such as the stability of the IVD segment, integrity and quality of the ECM, and
type, viability and activity of IVD cells. If a sufficient number of viable IVD cells is present, local application of a growth factor might be able to restore IVD homeostasis. Prolonged exposure to a bioactive substance can be achieved by gene therapy, or by a sustained release system. If cells in the IVD have lost their ability to synthesize a functional matrix, supplementation with cell populations may be a better option for effective IVD regeneration. In addition to their regenerative potential, transplanted cells may also have an immune-modulatory and/or anti-inflammatory potential, resulting in a reduction of pain.\textsuperscript{79}

A major challenge in regenerative therapies is determining which patients should be treated at what point in the degenerative cascade. Regeneration of a degenerated IVD would probably be most effective in a patient with early signs of IVD degeneration. But should we treat non-symptomatic patients?\textsuperscript{80} Should we only treat spinal segments that have the highest range of motion, and are more likely to cause clinical signs in future? The benefits of such a treatment should clearly outweigh the risks.

Each regenerative therapy faces its own biological issues and difficulties. A combination therapy, providing a delivery system, a bioactive substance, a specific cell type, and a system to temporary distract the IVD even might be the optimal treatment. Identification of the most effective combination of these therapies will require even more extensive research, but could hold great promise for functional repair of degenerated IVDs, and may replace current surgical treatments and their drawbacks for veterinary and human patients with low back pain.
References

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General discussion


Summary
The aim of the studies described in this thesis was to develop innovative treatments to regenerate degenerated intervertebral discs (IVDs) at an early stage in order to regain function and so prevent or delay further medical and surgical interventions in dogs and humans with low back pain.

Background
IVD disease is common in humans and dogs and is associated with IVD degeneration. In chapter 1 the current knowledge of the etiopathology of IVD degeneration in humans and dogs, as well as the treatments available, and evolving therapies are described. Current conservative and surgical treatment options typically address the signs of low back pain, without treating the underlying cause or restoring biomechanical function. Regenerative treatments seem to be most effective at an early stage of IVD degeneration and aim to restore homeostasis of the extracellular matrix (ECM), control inflammation, and prevent angio- and neurogenesis. Direct intradiscal injection via a minimal invasive technique is an elegant way to deliver bioactive agents, e.g. growth factors and pharmaceutical agents into the nucleus pulposus (NP). By encapsulating these bioactive agents in a biomaterial, e.g. microspheres or a hydrogel, they can be protected, and bioavailability and stability can be increased. However, materials, needle diameter, and injection volume need to be carefully selected when applied intradiscally, as they may also induce IVD degeneration.

Intradiscal application
PEA microspheres
In chapter 2 we showed a good cytocompatibility in vitro and biocompatibility in vivo in rabbits of polyester amide polymers (PEAs). Intradiscal injection of 40 μl of a sham solution through a 27G needle, and of PEA microspheres (PEAMs) through a 26G, could be safely applied in a canine model predisposed to IVD degeneration without accelerating degeneration over the course of 6 months. Sham-injected IVDs showed increased caveolin-1 expression compared with non-injected IVDs, possibly indicating increased cell senescence in sham-injected IVDs. PEAM-injected IVDs showed a significantly higher B-cell lymphoma 2-associated X/B-cell lymphoma 2 ratio compared with sham-injected IVDs, suggestive of an anti-apoptotic effect of the PEAMs. However, both findings were not supported by other analyses (clinical signs, disc height index, T2 values, biomolecular and biochemical analyses, and IVD histopathology).

Growth factor rhBPM-7
Recombinant human bone morphogenetic protein-7 (rhBMP-7) appears to be a promising growth factor for IVD regeneration, as it has been shown to have beneficial effects on extracellular matrix production of rabbit, bovine, and human IVD cells in vitro. In chapter 3
we showed this anabolic effect of rhBMP-7 on ECM production of canine NP cells isolated from early degenerated IVDs in vitro. However, in vivo intradiscal administration of a wide dose range of rhBMP-7 (2.5 – 250 μg) in spontaneously early degenerated canine IVDs, showed no regenerative effects at the IVD level. In fact, injection of 250 μg rhBMP-7, and to a lesser extent 25 μg rhBMP-7, resulted in extensive extradiscal bone formation. In order to intradiscally apply growth factors, such as rhBMP-7, they should be incorporated in sustained release systems, to increase bioavailability and to decrease the risk of side effects.

**pNIPAAM MgFe-LDH hydrogel loaded with an anti-inflammatory agent**

As described in chapter 1 several inflammatory mediators, including prostaglandin E₂ (PGE₂), have been investigated for their role in the catabolic processes of IVD degeneration. Oral administration of selective cyclooxygenase-2 (COX-2) inhibitors has been shown to reduce PGE₂ production and relieve low back pain, but have also been associated with systemic side effects. Intradiscal delivery of COX-2 inhibitors, like celecoxib (CXB), has been suggested as an alternative route of administration to avoid these systemic adverse reactions and enhance local efficacy. Incorporation of these drugs in a sustained release system, such as a hydrogel, is an attractive alternative to a bolus injection, as a higher loading dose and long term delivery can be accomplished by a minimal intradiscal intervention. Temperature sensitive poly-N-isopropylacrylamide (pNIPAAM)-based hydrogels are liquid at room temperature, and hence injectable through small needle diameters, but form a solid gel at 37°C, making them particularly suitable for intradiscal injection. In chapter 4 sustained release from CXB-loaded pNIPAAM MgFe-LDH hydrogels was demonstrated to suppress PGE₂ levels in the presence of TNF-α in vitro for 28 days. We demonstrate good biocompatibility and safety of this hydrogel after subcutaneous application in mice and intradiscal application in dogs predisposed to IVD degeneration. Sustained release of CXB from this hydrogel resulted in a limited inhibition (≈35%) of PGE₂ levels in the canine model. Based on low percentages of COX-2 positive cells at immunohistopathology, the lack of PGE₂ inhibition by the CXB-loaded pNIPAAM MgFe-LDH hydrogel might have been attributed to the limited presence of inflammation-induced COX-2. Although MR images, macroscopic and histologic parameters corroborated a degenerative status of the IVD, this early phase of spontaneous IVD degeneration, without clinical signs, appeared not to be associated with increased PGE₂ levels.

**Targeting inflammation in IVD degeneration**

The results of sustained release of CXB from the CXB-loaded pNIPAAM MgFe-LDH hydrogel in the canine model in chapter 4 raised the important question if increased levels of PGE₂ might only become evident in patients with clinical signs of IVD disease. Therefore, in
chapter 5 we determined the levels of PGE$_2$, but also of cytokines, chemokines, and matrix components in IVDs from chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs with and without clinical signs of IVD disease and correlate these to degeneration grade or herniation type. In addition, we investigated COX-2 expression in histological IVD samples of CD and NCD dogs. PGE$_2$ levels were significantly higher in the NP of degenerated IVDs compared with non-degenerated IVDs, and in herniated IVDs from NCD dogs compared with non-herniated IVDs of NCD dogs. COX-2 expression in the NP and annulus fibrosus (AF) and numbers of macrophages in the AF increased with advancing degeneration stages. Cytokines interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, immune protein-10, tumor necrosis factor-α, and granulocyte macrophage colony-stimulating factor were not detectable. Chemokine (C-C motif) ligand 2 (CCL2) levels in the NP from extruded disc samples were significantly higher compared with CCL2 levels in the AF of these samples and in the NP from protrusion samples. In contrast to findings described in literature, glycosaminoglycan content in our study showed no significant changes with degeneration grade or herniation type. Clinical efficacy of intradiscal application of sustained release systems loaded with an anti-inflammatory drug should be evaluated in canine patients with clinical signs that do not have an indication for surgical decompression.

Stabilization and unloading of the degenerative intervertebral disc

Degenerative lumbosacral stenosis (DLSS) is a common problem in large breed dogs, which is associated with IVD degeneration and herniation (Hansen type II). In dogs with severe signs of DLSS that do not improve with conservative treatment, surgical intervention with dorsal laminectomy is required. However, in some patients decompressive surgery alone can worsen LS instability, resulting in recurrence of clinical signs in 17 – 38% of the cases. Pedicle screw-rod fixation (PSRF) and interbody spinal fusion can provide stability of the lumbosacral junction and restore and maintain disc space height. In chapter 6, the long term clinical outcome of PSRF and lumbosacral spinal fusion were assessed in 12 client-owned dogs with severe DLSS. Clinical evaluation, force plate data, and owner questionnaires showed resolution or improvement of clinical signs in 67% and 33% of the cases, respectively. Although interbody vertebral bone fusion of the LS junction was not achieved by PSRF, this technique offers a surgical treatment option for large breed dogs with severe DLSS in which no other treatment is available.

As described in chapter 6, PSRF provided stability of the LS junction, but did not induce regeneration of the degenerated IVD. In the field of osteoarthritis, temporary joint distraction has been investigated as a relatively new approach to regenerate cartilage. Also, temporary distraction in rabbit models with induced IVD degeneration demonstrated signs of IVD repair. In chapter 7, we assessed the safety and efficiency of temporary (3-month) segmental distraction by using a PSRF device in a dog with clinical signs of DLSS
that was unresponsive to conservative management. Temporary PSRF in combination with
distraction was shown to be safe, to improve clinical signs, and to retain IVD height at 9
months follow-up. Clinical efficacy and regenerative effects of temporary pedicle screw-
rod fixation in order to distract and thereby unloading the early degenerated IVD should
be assessed in a larger cohort of canine patients with degenerative lumbosacral stenosis.

**Results and considerations for future studies**

The work presented in this thesis provides important information on intradiscal injection
itself, and intradiscal application of rhBMP-7 and two types of biomaterials, in a canine
model susceptible to IVD degeneration. Targeting PGE2 levels by intradiscal delivery of
sustained release systems loaded with the selective COX-2 inhibitor, CXB, seem most
beneficial in dogs that present with clinical signs. PSRF as a treatment for large breed dogs
with severe DLSS is shown to offer long term benefits. Furthermore, PSRF in combination
with temporary distraction is shown to be safe, improve clinical signs and to retain IVD
height in a canine patient with early signs of DLSS. The results of all studies, together with
scientific challenges and future perspectives are discussed in chapter 8.
Key findings

- Intradiscal injection of 40 μl of a sham solution through a 27G needle, and of polyester amide polymer microspheres through a 26G, can be safely applied in a canine model predisposed to IVD degeneration without accelerating degeneration over the course of 6 months.
- The growth factor recombinant human bone morphogenetic protein-7 (rhBMP-7) shows regenerative effects on canine disc cells isolated from early degenerated intervertebral discs (IVDs) in vitro.
- In vivo intradiscal administration of a wide dose range of rhBMP-7 (2.5 – 250 μg) in spontaneously early degenerated canine IVDs, shows no regenerative effects at the IVD level, but results in extensive extradiscal bone formation after injection of 25 and 250 μg rhBMP-7.
- Celecoxib (CXB)-loaded poly-N-isopropylacrylamide (pNIPAAM) MgFe-layered double hydroxide (LDH) hydrogels effectuates controlled release of CXB and sustained suppression of prostaglandin E₂ (PGE₂) levels in the presence of a pro-inflammatory agent in vitro for 28 days.
- The pNIPAAM MgFe-LDH hydrogel is biocompatible and safe to use for intradiscal delivery in dogs predisposed to IVD degeneration.
- Sustained release from CXB from the CXB-loaded pNIPAAM MgFe-LDH hydrogel results in limited inhibition (=35%) of PGE₂ levels in a canine model with early and mild IVD degeneration.
- PGE₂ levels and cytokine levels in degenerated and herniated tissues are significantly higher compared with non-degenerated and non-herniated tissues.
- Expression of the enzyme responsible for PGE₂ production in the nucleus pulposus (NP) and annulus fibrosus (AF) and numbers of macrophages in the AF increases with advancing degeneration stages indicating that inflammation plays a role in IVD degeneration.
- Pedicle screw-rod fixation (PSRF) offers a surgical treatment option for large breed dogs with severe degenerative lumbosacral stenosis in which no other effective treatment is available.
- PSRF alone does not result in interbody vertebral bone fusion.
- Temporary distraction of the IVD with the aid of PSRF is safe, improves clinical signs, and retains IVD height at 9 months follow-up.
List of abbreviations
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACAN</td>
<td>aggrecan</td>
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<tr>
<td>ADAMTS5</td>
<td>a disintegrin and metalloproteinase with thrombospondin motifs 5</td>
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<td>AF</td>
<td>annulus fibrosus</td>
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<td>AIC</td>
<td>Akaike information criterion</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>ASCs</td>
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<td>ASP</td>
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<td>ATDC5</td>
<td>AT805 teratocarcinoma derived chondrogenic cells</td>
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<td>BAX</td>
<td>B-cell lymphoma 2-associated X</td>
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<td>B-cell lymphoma-2</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BMM</td>
<td>Biomedical materials institute</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>BMPR1A</td>
<td>BMP receptor 1A</td>
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<td>DEC</td>
<td>Dierexperimentencommissie</td>
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<td>DHI</td>
<td>disc height index</td>
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<tr>
<td>DLSS</td>
<td>degenerative lumbosacral stenosis</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMBB</td>
<td>dimethylmethylenine blue</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EP</td>
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<tr>
<td>FA</td>
<td>ferulic acid</td>
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<tr>
<td>FasL</td>
<td>fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FSE</td>
<td>fast spin echo</td>
</tr>
</tbody>
</table>
FOV  field of view  
FPA  force plate analysis  
Fy'  peak propulsive force  
Fy'  peak braking force  
Fz'  peak vertical force  
G  complex modulus  
G'  storage modulus  
G"  loss modulus  
GAG  glycosaminoglycan  
GAPDH  glyceraldehyde 3-phosphate dehydrogenase  
GDF-5  growth and differentiation factor-5  
GM-CSF  granulocyte-macrophage colony-stimulating factor  
HA  hyaluronic acid  
hg-DMEM  high-glucose Dulbecco’s modified Eagle’s medium  
HPRT  hypoxanthine-guanine phosphoribosyl-transferase  
HR  hazard ratio  
ID1  inhibitor of DNA binding 1  
IDiDAS  New Early Therapies for Intervertebral Disc Diseases. Drug Delivery and Augmentation through Smart Polymeric Biomaterials  
IFN-γ  interferon gamma  
IGF-1  insulin-like growth factor-1  
IL  interleukin  
IL-1R  interleukin-1 receptor  
IL-1Ra  interleukin-1 receptor antagonist  
IM  intramuscular  
ITS  insulin-transferrin-selenium  
IV  intravenous  
IVD  intervertebral disc  
LBP  low back pain  
LCST  lower critical solution temperature  
LDH  layered double hydroxide  
LS  lumbosacral  
M  mean  
MMP13  matrix metalloproteinase 13  
MPCs  mesenchymal progenitor cells  
MR(I)  magnetic resonance (imaging)  
MRS  magnetic resonance spectroscopy  
MSCs  mesenchymal stem cells  
NA  not available  
NC  notochordal cells  
NCD  non-chondrodystrophic  
NGF  nerve growth factor  
NOG  noggin  
NPC  nucleus pulposus cells  
NP  nucleus pulposus  
NSAIDs  non-steroidal anti-inflammatory drugs  
OA  osteoarthritic  
P2  passage 2  
PBS  phosphate buffered saline
<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PEA</td>
<td>polyester amide polymers</td>
</tr>
<tr>
<td>PEAM</td>
<td>polyester amide polymer microspheres</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<tr>
<td>PLA</td>
<td>poly lactic acid</td>
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<tr>
<td>PLGA</td>
<td>poly lactic-co-glycolic acid</td>
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<tr>
<td>pNIPAAM</td>
<td>poly-N-isopropylacrylamide</td>
</tr>
<tr>
<td>PO</td>
<td>per os</td>
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<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>p/s</td>
<td>penicillin/streptomycin</td>
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<td>PSRF</td>
<td>pedicle screw-rod fixation</td>
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<tr>
<td>P/T</td>
<td>pelvic/thoracic</td>
</tr>
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<td>PTGES</td>
<td>prostaglandin E synthase</td>
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<td>qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
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<tr>
<td>BMP-7</td>
<td>bone morphogenetic protein-7</td>
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<tr>
<td>rh</td>
<td>recombinant human</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
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<td>RPS19</td>
<td>ribosomal protein S19</td>
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<td>S</td>
<td>signal intensity</td>
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<td>standard deviation</td>
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<td>SDHA</td>
<td>succinate dehydrogenase complex, subunit A, flavoprotein variant</td>
</tr>
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<td>T</td>
<td>brachyury</td>
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<tr>
<td>T₁p</td>
<td>application of T₁ in the rotating frame</td>
</tr>
<tr>
<td>T₁-TFE</td>
<td>T₁-turbo field echo</td>
</tr>
<tr>
<td>T₂W</td>
<td>T₂-weighted</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>echo time</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>TIMP₁</td>
<td>tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
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<td>TSE</td>
<td>turbo-spin echo</td>
</tr>
<tr>
<td>TSL</td>
<td>spin-lock time</td>
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Dutch summary/
Nederlandse samenvatting
Samenvatting

Het doel van dit proefschrift was het ontwikkelen van innovatieve behandelingen om de vroeg gedegenereerde tussenwervelschijf (TWS) te regenereren en zo de functie te herstellen. Hierdoor zou de toepassing van palliatieve medicamenteuze en/of chirurgische behandelingen in zowel de hond als de mens kunnen worden voorkomen of vertraagd.

Achtergrond

Aandoeningen van de tussenwervelschijf worden veelvuldig gezien in zowel de hond als de mens en zijn geassocieerd met degeneratie van de TWS. Hoofdstuk 1 bespreekt de huidige literatuur met betrekking tot het proces van TWSdegeneratie. Daarnaast wordt ingegaan op de behandelingen die momenteel beschikbaar zijn, maar ook die in ontwikkeling zijn. De huidige conservatieve en chirurgische behandelmogelijkheden zijn vooral palliatief en leiden niet tot herstel van de TWS zelf. Regeneratieve behandelingen lijken het meest effectief in een vroeg stadium van TWSdegeneratie. Zij hebben als doel de homeostase in de TWS te herstellen, een ontstekingsproces te beheersen en vaat- en zenuwvinger te voorkomen. Intradiscale injectie met behulp van een minimaal invasieve techniek, biedt de mogelijkheid bioactieve stoffen, zoals groeifactoren en geneesmiddelen, direct in de kern van de TWS, de nucleus pulposus (NP) af te leveren. Door deze bioactieve stoffen in een biomateriaal, zoals microsferen of een hydrogel, te incorporeren kan bescherming worden geboden en kan de biologische beschikbaarheid en stabiliteit van deze stoffen worden verhoogd. Voor intradiscale injectie is de keuze van het biomateriaal, de diameter van de naald en het injectievolume van groot belang, aangezien deze factoren zelf ook TWSdegeneratie kunnen induceren.

Intradiscale injectie

PEA microsferen

In hoofdstuk 2 werd een goede celcompatibiliteit in vitro en biocompatibiliteit in vivo in konijnen aangetoond van polyester amide polymeren (PEAs). Een volume van 40 μl van een sham conditie via een 27G naald en van PEA microsferen (PEAM) via een 26G naald kon veilig intradiscal worden geïnjecteerd in een hondenmodel gepredisposeerd voor TWSdegeneratie. Tijdens de vervolgperiode van 6 maanden werd geen toename van degeneratie gezien. Wel werd een verhoogde expressie van caveolin-1 in sham-geïnjecteerde TWSen in vergelijking met niet-geïnjecteerde TWSen gezien, wat zou kunnen wijzen op een verhoogde cel senescentie in sham-geïnjecteerde TWSen. In PEAM-geïnjecteerde TWSen werd een significant hogere B-cell lymphoma 2-associated X/B-cell lymphoma 2 ratio gezien dan in sham-geïnjecteerde TWSen, wat suggestief zou kunnen zijn voor een anti-apoptotisch effect van de PEAMs. Echter, alle andere analyses (klinische verschijnselen, tussenwervelschijfhoogte index, T2 waarden,
biomoleculaire en biochemische analyses en histologisch onderzoek van de TWSen) ondersteunden deze twee bevindingen niet.

**Groeifactor rhBMP-7**

Recombinant human bone morphogenetic protein 7 (rhBMP-7) is in de literatuur beschreven als een veelbelovende groeifactor voor TWS regeneratie. *In vitro* is aangetoond dat het een gunstig effect heeft op de aanmaak van extracellulaire matrix (ECM) door TWS cellen afkomstig van konijnen, runderen en mensen. In *hoofdstuk 3* werd dit effect van rhBMP-7 *in vitro* ook gedemonstreerd in NP cellen afkomstig van honden met vroeg gedegenereerde TWSen. Intradiscale injectie van een brede range van doseringen van rhBMP-7 (2.5 – 250 μg) *in vivo*, in een hondenmodel gekenmerkt door spontane en vroege TWSdegeneratie, liet echter geen regeneratie van de TWSen zien. Sterker nog, injectie van 250 μg rhBMP-7 en in mindere mate bij 25 μg rhBMP-7, liet uitgebreide botnieuwvorming rond de TWSen zien. Het verdient dan ook aanbeveling om groeifactoren, zoals rhBMP-7, voor intradiscale toepassing te incorporeren in gecontroleerde afgifte systemen, om zo de biologische beschikbaarheid te verhogen en de risico’s op bijwerkingen te verlagen.

**pNIPAAM MgFe LDH-hydrogel geladen met een ontstekingsremmer**

Zoals beschreven in *hoofdstuk 1* zijn er meerdere pro-inflammatoire mediatoren, waaronder PGE2, onderzocht voor hun rol in het degeneratieve proces in de TWS. Orale toediening van een selectieve cyclooxygenase-2 (COX-2) remmer, zoals celecoxib (CXB), kan de PGE2 productie verlagen en zo lage rugpijn effectief bestrijden, maar kan ook leiden tot systemische bijwerkingen. Een geschikt alternatief is de intradiscale toepassing van een COX-2 remmer, waarmee systemische bijwerkingen kunnen worden vermeden en de lokale effectiviteit kan worden verhoogd. Incorporatie van deze medicijnen in een gecontroleerd afgifte systeem, zoals bijvoorbeeld een hydrogel, heeft de voorkeur boven een bolusinjectie, aangezien een hogere dosering kan worden geladen en een langdurige afgifte kan worden bewerkstelligd middels een minimaal invasieve procedure. Temperatuurgevoelige poly-N-isopropylacrylamide (pNIPAAM) hydrogels zijn uitermate geschikt voor intradiscale injectie doordat ze vloeibaar zijn bij kamertemperatuur en dus door naalden met een hele kleine diameter geïnjecteerd kunnen worden, maar veranderen in een stevige gel bij lichaamstemperatuur (37 °C). In *hoofdstuk 4* werd aangetoond dat gecontroleerde afgifte van een CXB-geladen pNIPAAM MgFe-layered double hydroxide (LDH) hydrogel, PGE2 spiegels kon onderdrukken in de aanwezigheid van tumor necrosis factor-α (TNF-α) *in vitro* gedurende 28 dagen. De gel kon veilig subcutaan in muizen en intradiscaal in honden gepredisponeerd voor TWSdegeneratie worden toegepast en liet een goede biocompatibiliteit zien. Gecontroleerde afgifte van CXB resulteerde in het hondenmodel in een verlaging van PGE2 spiegels van slechts ≈35%.
Gezien het eveneens lage gehalte aan COX-2 positieve cellen dat met immunopathologie werd gezien, werd deze marginale daling van PGE₂ spiegels gewijd aan de beperkte aanwezigheid van ontsteking geïnduceerde COX-2. Ondanks het feit dat zowel de MRI beelden als de macroscopische en histologische parameters duidden op degeneratie van de TWSen, lijkt deze vroege fase van TWSdegeneratie in honden zonder klinische verschijnselen, niet gepaard te gaan met verhoogde PGE₂ spiegels.

**Aanpak van ontstekingsmediatoren in tussenwervelschijfdegeneratie**

De resultaten van de gecontroleerde afgifte van CXB uit de CXB-geladen pNIPAAM MgFe-LDH hydrogel in het hondenmodel in hoofdstuk 4 wierpen de belangrijke vraag op of er wellicht alleen sprake is van verhoogde PGE₂ spiegels in patiënten met klinische verschijnselen van TWSdegeneratie. Om dit inzichtelijk te maken werden in hoofdstuk 5 spiegels van PGE₂, cytokines, chemokines en matrix componenten bepaald in TWSen van chondrodystrofe (CD) en niet-chondrodystrofe (NCD) honden, welke werden gecorrereerd aan degeneratie graad of type hernia. Daarnaast werd de expressie van COX-2 in histologische monsters van CD en NCD honden onderzocht. PGE₂ spiegels bleken significant hoger te zijn in de NP van gedegenereerde TWSen in vergelijking met niet-gedegenereerde TWSen, en in gehernieerde TWSen in vergelijking met niet-gehernieerde TWSen in NCD honden. Een stijging van de COX-2 expressie in de NP en AF en het aantal macrofagen in de AF werd gezien naarmate de graad van degeneratie toenam. Cytokines interleukine (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, immune protein-10, TNF-α en granulocyte macrophage colony-stimulating factor werden niet aangetroffen. Chemokine (C-C motif) ligand 2 (CCL2) spiegels in de NP van geëxtrudeerde TWSen waren significant hoger vergeleken met CCL2 spiegels in de AF van deze TWSen en in de NP van geprotrudeerde TWSen. In tegenstelling tot wat beschreven is in de literatuur, werd geen significante verandering in het gehalte aan glycosaminoglycanen waargenomen naarmate de degeneratie graad toenam of met het type hernia. De klinische werkzaamheid van de intradiscale toepassing van gecontroleerde afgifte systemen geladen met ontstekingsremmers, kan het beste geëvalueerd worden in honden met klinische klachten.

**Stabilisatie en distractie van de gedegenereerde tussenwervelschijf**

Een regelmatig voorkomende aandoening in grote honden is degeneratieve lumbosacræale stenose (DLSS), waarbij TWSdegeneratie en herniëring (Hansen type II) een belangrijke rollen te spelen. In honden met ernstige verschijnselen van DLSS, waarbij een conservatieve behandeling onvoldoende of geen verbetering laat zien, is een chirurgische behandeling, bestaande uit een dorsale laminectomie, geïndiceerd. In 17 – 38% van de patiënten die deze behandeling hebben ondergaan wordt echter een terugval van klinische verschijnselen gezien, als gevolg van instabiliteit van de lumbosacræale overgang. Met behulp van pedikelschroef-staaffixatie (PSSF) en spinale fusie
kan stabiliteit ter hoogte van de lumbosacrale overgang worden gecreëerd en kan de hoogte van de TWS worden hersteld en behouden. In hoofdstuk 6 werd de klinische uitkomst op lange termijn van PSSF en spinale fusie van de lumbosacrale overgang geëvalueerd in 12 gezelschapshonden met ernstige DLSS. Klinische beoordeling, grondreactie krachten en enquêtes ingevuld door eigenaren wezen uit dat de klinische verschijnselen verdwenen of verbeterden in respectievelijk 67% en 33% van de honden. Ondanks het achterwege blijven van spinale fusie van de lumbosacrale overgang, werd aangetoond dat PSSF een geschikte chirurgische behandeling is voor grote honden met ernstige verschijnselen van DLSS, waarvoor geen andere behandelmogelijkheden beschikbaar zijn.

Hoewel PSSF stabiliteit aan de lumbosacrale overgang biedt zoals beschreven in hoofdstuk 6, leidt deze techniek niet tot regeneratie van de gedegenereerde TWS. Op het gebied van osteoarthritis wordt veel onderzoek verricht naar een relatief nieuwe methode waarbij tijdelijke gewrichtsdistractie wordt toegepast om zo regeneratie van het kraakbeen te stimuleren. In konijnenmodellen met geinduceerde TWSdegeneratie werden na tijdelijke distractie van de TWS tekenen van regeneratie gezien. In hoofdstuk 7 werd de veiligheid en effectiviteit van tijdelijke (3 maanden) distractie door de toepassing van PSSF beschreven in een hond met klinische verschijnselen van DLSS, waarbij een medicamenteuze behandeling onvoldoende effect gaf. De resultaten toonden aan dat tijdelijke distractie van de lumbosacrale overgang met behulp van PSSF een veilige techniek is, die leidt tot klinische verbetering en waarbij de hoogte van de TWS tot in ieder geval 9 maanden na de ingreep behouden blijft. De klinische werkzaamheid en regeneratieve effecten van tijdelijke PSSF om distractie te bewerkstelligen en zo de TWS te ontlasten, zal geëvalueerd dienen te worden in een grotere groep DLSS patiënten.

Resultaten en toekomstperspectieven
Dit proefschrift bevat belangrijke informatie over intradiscale injectie en de intradiscale toepassing van rhBMP-7 en twee typen biomaterialen in een hondenmodel gepredisponeerd voor TWSdegeneratie. Intradiscale toepassing van een gecontroleerd afgifte systeem geladen met de selectieve COX-2 remmer, CXB, lijkt het meest zinvol in honden met klinische verschijnselen. PSSF als een behandeling voor grote honden met DLSS is op de lange termijn effectief gebleken. Tevens is aangetoond dat PSSF in combinatie met tijdelijke distractie veilig kan worden toegepast, tot klinische verbetering leidt en de hoogte van de TWS kan behouden in een hond met milde klinische verschijnselen van DLSS. De resultaten van alle studies, evenals de wetenschappelijke uitdagingen en toekomstperspectieven worden besproken in hoofdstuk 8.
Belangrijkste bevindingen

- Intradiscale injectie van 40 μl van een sham door een 27G naald en van microsferen opgebouwd uit PEAs door een 26G naald, kan veilig worden toegepast in een hondenmodel gepredisponeerd voor tussenwervelschijf (TWS) degeneratie; degeneratie verergerde niet over een periode van 6 maanden.
- *In vitro* heeft de groeifactor rhBMP-7 regeneratieve effecten in NP cellen van honden geïsoleerd uit vroeg gedegenereerde TWSen.
- Intradiscale toepassing van uiteenlopende doseringen van rhBMP-7 (2.5 – 250 μg) in spontane vroeg gedegenereerde TWSen van honden, laat geen regeneratieve effecten zien op TWS niveau, maar resulteert na injectie van 250 en 25 μg rhBMP-7 in uitgebreide extradiscale botnieuwvorming.
- Celecoxib (CXB)-geladen poly-N-isopropylacrylamide (pNIPAAM) MgFe-layered double hydroxide (LDH) hydrogels bewerkstelligen gecontroleerde afgifte van celecoxib en onderdrukking van prostaglandine E₂ (PGE₂) niveaus in de aanwezigheid van een pro-inflammatoir agens *in vitro* gedurende 28 dagen.
- De pNIPAAM MgFe-LDH hydrogel is biocompatibel en veilig te gebruiken als intradiscale toepassing in honden gepredisponeerd voor TWS degeneratie.
- Gecontroleerde afgifte van CXB uit CXB-geladen pNIPAAM MgFe-LDH hydrogels resulteert in een beperkte onderdrukking (≈35%) van PGE₂ niveaus in een hondenmodel met vroege en milde TWSdegeneratie.
- PGE₂ en cytokine niveaus in gedegenereerde en gehernieerde weefsels zijn significant hoger vergeleken met niveaus in niet-gedegenereerde en niet-gehernieerde weefsels.
- Expressie van het enzym verantwoordelijk voor PGE₂ productie in de NP en AF en het aantal macrofagen in de AF neemt toe naarmate de degeneratie graad toeneemt, wat impliceert dat ontsteking een rol speelt in TWSdegeneratie.
- Pedikelschroef-staaffixatie (PSSF) biedt een chirurgische behandeloptie voor grote honden met ernstige degeneratieve lumbosacraal stenose, waarvoor geen andere effectieve behandeling meer beschikbaar is.
- PSSF alleen resulteert niet in spinale fusie.
- Tijdelijke distractie van de TWS met behulp van PSSF is veilig, verbetert klinische verschijnselen en zorgt voor behoud van de TWS ruimte gedurende een opvolgperiode van 9 maanden.
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Curriculum vitae
Nicole Willems was born on the 22\textsuperscript{nd} of February 1980 in Oude-Tonge, The Netherlands. After graduating from secondary school (R.G.O. Middelharnis) she started to study Veterinary Medicine at Utrecht University in 1999. During her veterinary training she was President of the Veterinary Student Association D.S.K. (2002-2003). She graduated with honors in 2007 and worked as a veterinary surgeon in private practice for 3 ½ years. In 2010 she returned to the Faculty of Veterinary Medicine at Utrecht University and completed a 1-year clinical rotating internship at the Department of Clinical Sciences of Companion Animals. Subsequently she started her PhD at the Division of Orthopedic surgery and Neurosurgery at the Faculty of Veterinary medicine in Utrecht and combined this with clinical work at the Department of Clinical Sciences of Companion Animals in Utrecht in 2015. She currently works as an ECVS (European College of Veterinary Surgeons) resident in small animal surgery at the Department of Clinical Sciences of Companion Animals at the Faculty of Veterinary Medicine at Utrecht University.