Utrecht University

Controlled release of Celecoxib: an in vitro study with canine degenerated nucleus pulposus cells

Research internship, Master Veterinary Medicine

Boegschoten, S. (Sofie)
15-5-2016
Supervisors: Mw. Drs A.R. Tellegen and mw. Dr. M.A. Tryfonidou

Table of Contents

Abstract 2
Introduction 3
Aim of this study 6
Materials and Methods 7
Pilot study design 7
Gene expression 8
Histology 10
Safranin-O / Fast Green staining 10
Biochemical analyses 11
Statistical analyses 11
Results 12
Gene expression 12
Safranin-O / Fast Green staining 14
Biochemical analyses 15
Discussion inflammatory model 16
Hydrogel study 18
Study design 18
Results 20
Gene expression 20
General Discussion 24
Conclusions 27
References 28

Abstract

Introduction- Degeneration of the intervertebral disc (IVD) is a complex process and is known to be a cause of different diseases associated with lower back pain in dogs.
Aim of the study- The overall purpose of this project is to translate treatment towards the veterinary patients suffering from lower back pain by a controlled release of medication via local delivery. The hypothesis of this *in vitro* study is that celecoxib released from a hydro-gel has a sustained anti-inflammatory effect compared to celecoxib bolus treatment on canine degenerated nucleus pulposus cells (NPCs).
Materials and methods- Canine NPCs were cultured in pellets and treated with TNF-α to induce inflammation to recreate a situation similar to the in vivo situation of a degenerated IVD. Pellets were treated with celecoxib loaded hydro-gel and different concentrations of celecoxib in bolus form. The effects were evaluated by performing gene measurement, biochemical analysis and histological staining.
Results- Pellets treated with TNF-α in qPCR, biochemical and histological analysis showed a decrease of anabolic matrix components (COL2 and ACAN) and an increase of the catabolic components (MMPs and ADAMTS5). Pellets treated with the loaded hydro-gel showed an even further downregulation of the anabolic genes and upregulation of the catabolic genes. Treatment with celecoxib bolus treatment did not show any regenerative effect.
Conclusion- No anti-inflammatory effect was shown of celecoxib in this study. Recreating an inflammatory model for an in vitro study to evaluate a treatment of degenerated intervertebral discs is complicated, mainly due to possible dominating effects of TNF-α and a dynamic process of inflammation and regeneration. Data shown in this study were not in line with published data of the use of celecoxib hydro-gel in vivo.

Introduction

The spine is composed of vertebral bodies which are each connected by an intervertebral disc (IVD). The IVDs have multiple functions, of which one is to operate as shock absorbers. Because of a cushion like mechanism it prevents the vertebral bodies against physical forces. IVDs allow movement of the spine and provide stability as well. The IVD is composed of multiple structures each providing their own function (Figure 1). The central part of the IVD is the nucleus pulposus (NP), a bean shaped structure. The NP is mainly composed of water which absorbs the compressing load during axial compressing. To protect the NP from tearing during these compressions it is surrounded with a dense network of fibrous lamellae, the annulus fibrosus (AF). The transition from the mucoid NP to the fibrous AF is called the transition zone (TZ). The cranial and caudal parts of the IVD are strongly connected to the vertebral bodies with the cartilaginous endplates (EPs) as well as by the outer layers of the AF.[1]

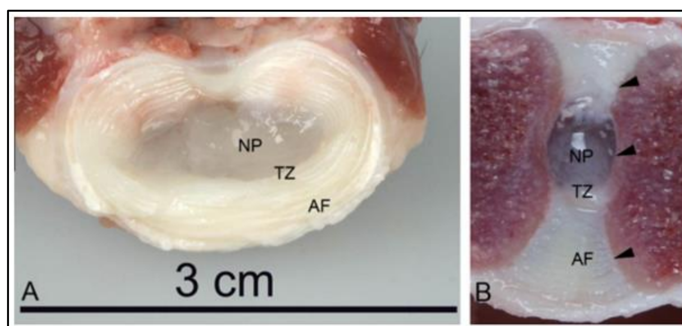

Figure 1 Transverse (A) and sagittal (B) sections through a L5-L6 intervertebral disc, showing the nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF) and endplates (arrowheads).[1]

Degeneration of the IVD is a complex process and is known to be a cause of different diseases associated with lower back pain in dogs. Degeneration is characterized by changes in the conformation of the cells and the extracellular matrix of the IVD. The specific pathophysiology of these changes is little described in dogs. Previous studies have shown that there are similarities to the disease process seen in human beings. The pathophysiology known in human IVD degeneration can serve as a model for the pathophysiology of IVD degeneration in dogs. In a review article IVD degeneration is described as 'an aberrant, cell-mediated response to progressive structural failure of the IVD. It is associated with genetic predisposition, chronic physicomechanical overload and trauma, inadequate metabolite and nutrient transport to and from the cells with the IVD matrix, cell senescence and death, altered levels of enzyme activity, changes in matrix macromolecules, and changes in water content.'[1]

Regarding the genetic predisposition a distinction can be made between chondrodystrophic (CD) and non-chondrodystrophic (NCD) dog breeds. The pathophysiology is similar in these two types of dogs. The genesis of IVD degeneration in these dog breeds can be distinguished by the age of onset, frequency as well as the location of the spine and different types of nucleus pulposus herniation it may lead to.[2]

However, the changes of the NP matrix seen in IVD degeneration appear to be comparable in both types of dogs. Because of these changes the matrix becomes more rigid, which can be explained by an increase in collagen type I content and decrease of the content of glycosaminoglycan (GAG), a side chain of proteoglycan. In a healthy NP a network of proteoglycan and collagen type II fibres create a strong osmotic gradient, attracting water leading to high intradiscal pressure. The resultant changes within the extracellular matrix caused by the degeneration have a number of consequences, resulting in loss of structural integrity, decreased hydration, and a reduced ability to withstand compression.[3] As a result to this the synthesis of a new healthy matrix is interfered with the decreased nutrition supply. This way it will lead to a vicious circle, a repeated structural/functional failure and inadequate repair of the IVD matrix (see Figure 2).[1] An important principle in this process is the fact that the healthy IVD is minimally innervated. Nerves as well as blood vessels approach only the outer layer of the AF.[4] Experiments of nutrient supply in healthy IVD in goats have shown that cartilage end-plates are responsible for the supply and excretion of the nutrients to and from the inner part of the NP.[5] Other studies have shown that NP cells respond to mechanical injury or inflammation by increasing the expression of neurotrophins and angiogenic factors.[4]

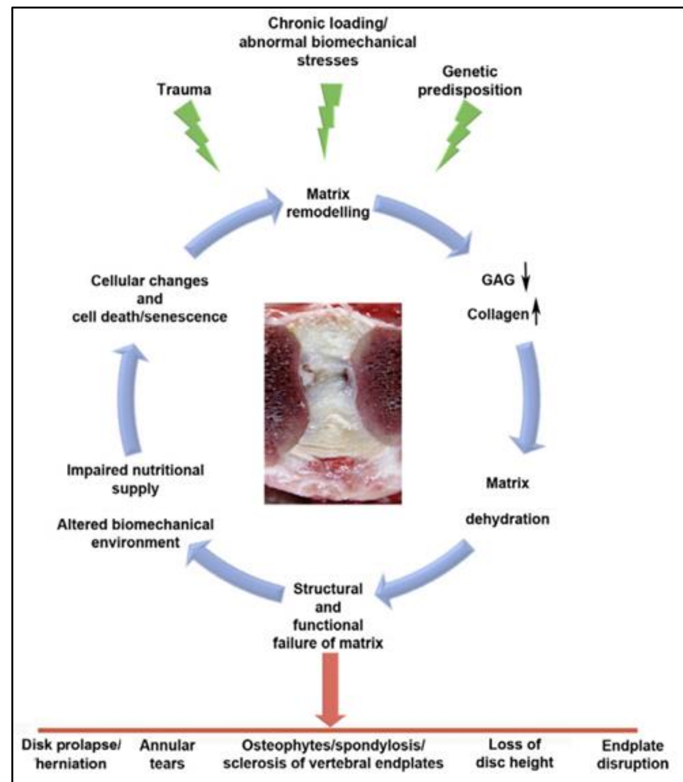

Figure 2 Schematic representation of the pathophysiology of intervertebral disc degeneration. Indicating the vicious circle occurring in the pathological process. Showing the factors and resulting in a cascade changes resulting in degeneration. The repeated structural/functional failure and inadequate repair of the intervertebral disc matrix makes this an ongoing process. Several factors (green symbols) may initiate or affect/accelerate the degenerative cycle, and structural/functional failure may result in various structural changes (red arrow) of the intervertebral disc and adjacent vertebral bodies. [1]

As mentioned before changes in enzyme activity are seen in the process of IVD degeneration. In a healthy IVD matrix, the anabolic and catabolic processes are regulated by enzymes as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Disturbance of the balance of the process by these enzymes induces a degradation of the IVD matrix.[3] A study associated these enzymes and degradation products with the pain seen in IVD degeneration. The authors believed that because of annular fissures extending from the nucleus to the outer annulus, the nerve endings are exposed to these degradation products. As well as this neural factor the combination with

mechanical loading and inflammatory factors is seen as a cause of discogenic pain. Thus, the disc itself behaves as the primary pain generator in patients with IVD degeneration.[6] Inflammatory factors which are known to contribute to the degeneration process are inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6.[1]

These inflammatory mediators are an important target of a potential therapy to slow the disease progress and thereby treat the discogenic pain.[7] Anti-inflammatory treatment has been studied in several inflammatory diseases, but for treating discogenic pain little evidence of its efficacy exists. Studies performed in rodent models show that antagonizing TNF-α, nerve growth factor and IL-6, interleukin produced an analgesic effect. These may be promising analgesic treatment options for IVD degeneration.[8] Alongside the analgesic effect, treatment may stop further degeneration by interrupting the vicious circle. Eventually, treatment may ideally provide regeneration of the IVD.

A non-steroidal anti-inflammatory drug (NSAID) approved for pain management in humans is celecoxib. The working mechanism of this drug is inhibiting a key enzyme cyclo-oxygenase-2 (COX-2). Cox-2 is part of the pathway of prostaglandin E2 (PGE2) biosynthesis (see Figure 3). Arachidonic Acid (AA) is released when cell damage occurs, due to mechanical damage, cytokines or other stimuli. [9] This AA is converted by COX-1 or COX-2 into prostaglandin H2 (PGH2). The conversion from PGH2 to prostaglandin E2 (PGE2) is catalyzed by the microsomal enzymes prostaglandin E synthase (PGES).[10] In non-inflammatory condition AA is first converted by COX 1 into PGH2 and further converted in a basal level of PGE2 catalyzed by prostaglandin E synthase 2 (PGES2). This basal PGE2 has physiological functions in several organs. In inflammatory conditions the increased amount of AA is converted by COX-2 and then a pathologic amount of PGE2 is catalyzed by the enzyme prostaglandin E synthase 1(PGES1). [11]

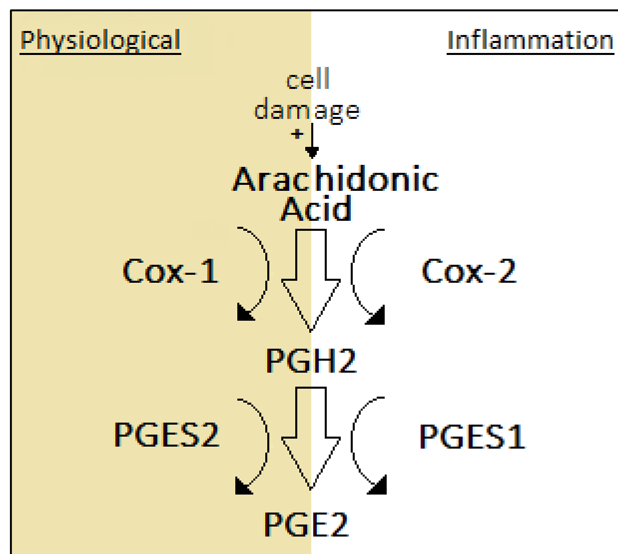

Figure 3 Pathway of prostaglandin E2 biosynthesis. Starting with the occurrence of cell damage which upregulated the production of Arachidonic Acid. Within physiological situation the main involving content is cyclooxygenase 1 (COX-1) and prostaglandin E synthase 2 (PGES2). During the inflammatory process cyclooxygenase 2 (COX-2) and prostaglandin E synthase 1 (PGES1) are predominant.

Given the fact that in IVD degeneration inflammation plays an essential role, celecoxib may be a suitable treatment option in dogs with IVD degeneration. However, concerns have risen about the systemic toxicity of celecoxib. Although it induces fewer side effects in the gastrointestinal tract compared with other (non-selective COX-2) NSAIDs[12], prolonged use in combination with a relative high dosage increases the risk for cardiovascular problems.[13] Also

earlier mentioned, poor innervation of the IVD makes it questionable if systemic provided therapies are able to reach the centre part of the IVD. To avoid this, formulations which are able to locally release drugs for a prolonged period ideally result in therapeutically effective local concentrations while plasma concentrations remain significantly low. Furthermore, patient compliance will improve compared to daily administration.[14]

An example of this form of drug delivery system is acetyl-capped poly (ε -caprolactone-co-lactide)-poly (ethylene glycol)-poly(ε -caprolactone-co-lactide) (PCLA-PEG-PCLA) based gels. These gels are easy to inject and when reaching body temperature the viscosity is changed in a more consistent gel (see Figure 4). Thereby these gels are suitable for loading and releasing hydrophilic drugs, like celecoxib. This combination has already been tested in rats and horses in the treatment of osteoarthrosis.[13,14] These studies showed that injection in joints results in high local celecoxib concentrations for a prolonged time, while significantly low systemic exposure was seen. This prolonged release is based on both diffusion and chemical degradation[13], which takes around 12 weeks in vitro and 8 weeks in vivo.[14] These gels are therefore attractive delivery systems for local drug administration which generate high local doses without the risk of side effects associated with oral administration and have therefore great potential for clinical applications in IVD degeneration. However, rightly pointed out by Walter et al., in the clinical situation, anti-inflammatory therapy would likely be introduced into an inflamed environment, given the previous mentioned inflammatory component to IVD disease. It is therefore a priority to investigate if the efficacy of the treatment is negatively influenced by an existing inflammatory state.[7]

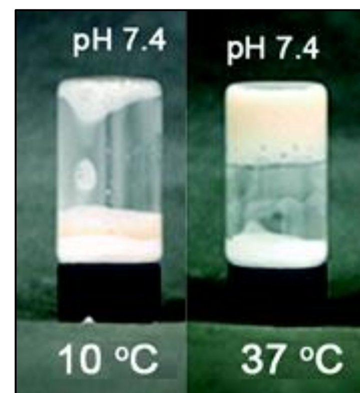

Figure 4 poly(ε -caprolactone-co-lactide)-poly (ethylene glycol)-poly (ε -caprolactone-co-lactide). Increase of viscosity from aqueous solution at 10°C to a solid gel at 37°C / body temperature. [30]

Aim of this study

The overall purpose of this project is to translate novel treatments towards the veterinary patients suffering from back pain by a controlled release of medication via local delivery. The specific aim of this *in vitro* study is to determine the anti-inflammatory effect of celecoxib, processed in a thermo-gel drug delivery system, on degenerated canine nucleus pulposus cells. It is hypothesized that celecoxib released from this delivery system has a sustained anti-inflammatory effect.

Materials and Methods

This present study was subdivided in a pilot study and the final hydrogel study. The pilot study was first performed to examine the inflammatory effect of Tumor Necrosis Factor α (TNFα) on nucleus pulposus cells and the effect of a bolus treatment with celecoxib. The results of this pilot study were taken in account by designing the final hydrogel study. The described materials and methods were equally for both studies. Study design and results are displayed separately.

Pilot study design

This pilot study was performed to examine the inflammatory effect of Tumor Necrosis Factor α (TNF-α) on nucleus pulposus cells and the effect of a bolus treatment with celecoxib. The design of this study is shown in Figure 5.

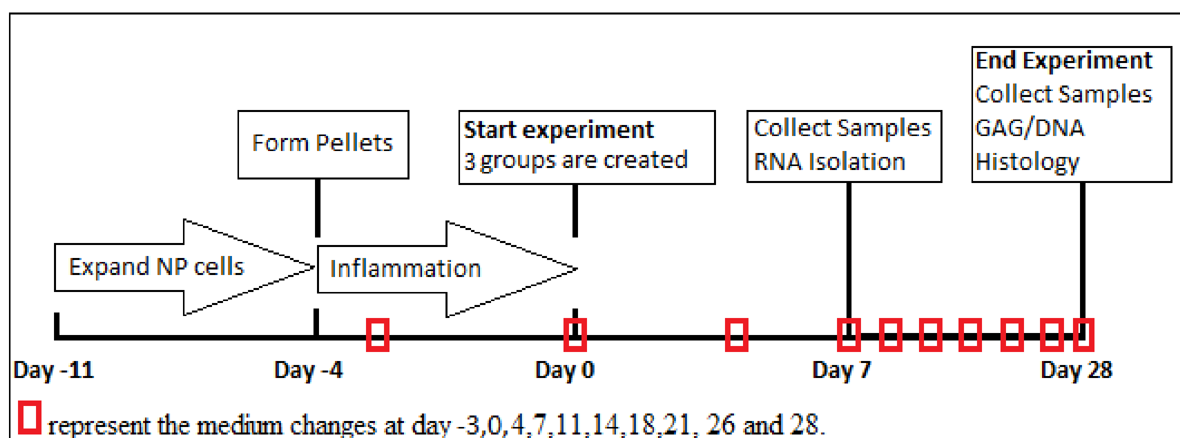

Figure 5. Design of pilot study plotted over time. During the first seven days the pooled nucleus pulposus cells were expanded. After pellets were formed inflammation was induced in all conditions, except the control group, by adding Tumor Necrosis Factor α (TNF-α). At day 0 the experiment started and three different condition groups were created (see Table 1). At day 7 samples were collected in triplicate for gene expression measurements. At day 28 the experiment ended and the samples were used for biochemical analysis and histological staining.

Expanding Nucleus pulposus cells

Nucleus pulposus cells were obtained of four different NCD dogs. These cells were pooled and expanded. During seven days the cells were cultured in an expansion medium containing DMEM, high glucose, GlutaMAX$^{(TM)}$, pyruvate (Invitrogen, 31966), FBS (High performance, Gibco, 16000-044), Penicillin/Streptomycin (PAA Laboratories, P11-010), Ascorbic acid 2-phosphate (Sigma, A8960), Dexamethasone (Sigma, D1756), bFGF (AbD Serotec, PHP105), Fungizone (15290-018, Invitrogen).

Groups	Chondrogenic medium + TGF- β	TNFα	Bolus CXB
1. Control	+		
2. TNF-α	+	+	
3. TNF-α CXB	+	+	+

Table 1 Additives at day 0 per group. *Group 1*: Only cultured in medium with 5 ng/ml TGF- β (negative control). *Group 2:* 10 ng/ml TNF-α was added to achieve the inflammatory effect (positive control). *Group 3:* 10^-7M celecoxib was added to evaluate the anti-inflammatory effect of celecoxib.

Forming Pellets and inducing inflammation
After the expansion phase, cells were collected and divided in pellets existing of 200.000 nucleus pulposus cells each. Pellets were treated with TGF-β to support the cells to remain vital. Thereafter inflammation was induced in all pellets, except the pellets in the control group, by adding TNF-α daily. During this period of five consecutive days chondrogenic medium was added to the pellets, containing DMEM, high glucose, GlutaMAX(TM), pyruvate (Invitrogen, 31966), ITS+ premix (Corning, 354352), L-Proline (Sigma, P5607), Penicillin/Streptomycin (PAA Laboratories, P11-010), Fungizone (15290-018, Invitrogen), TGF-β, Ascorbic acid 2-phosphate (Sigma, A8960), Bovine Serum Albumin (Sigma, A9418) medium change twice a week, first five days TNF- α daily, thereafter during each medium change.

Gene expression

Gene expression levels were analyzed in triplicate on samples collected at day 7. The genes of interest can be divided in four different expression groups: matrix production/anabolism, matrix remodeling and degradation/catabolism, inflammation and apoptosis.

RNA was extracted by crushing the pellets using pestles (P9951-901, Argos Technologies, Elgin, USA) after the pellets were frozen in liquid nitrogen. This step was repeated several times till pellets were completely crushed. 350 µl RLT (151012987, Qiagen, Hilden, Germany) + β-mercaptoethanol were added to the samples and mixed. This lysis buffer ensures that the cell membranes were digested and the RNA would become available for further isolation. The total content was placed in a new microtube and frosted.

350µl 70% ethanol was added to the defrosted and centrifuged samples. The content was transferred in RNeasy micro spin columns (74004, Qiagen, Valencia, USA) and were centrifuged for 30 seconds at maximum speed. The flow-through was removed. This step was repeated with 350 µl RW1 buffer (151013997, Qiagen, Hilden, Germany) to wash the columns. After the washing step, 80 µl DNAse (RNAse-Free DNase Set, 79254, Qiagen, Valencia, USA) was added on the membrane and 15 minutes incubating time was taken. With this step DNA in the samples was deleted so only RNA would remain. The washing step was repeated. 500 µl RPE^{+}(151014626, Qiagen, Hilden, Germany) was added in the columns after they were placed in a new collection tube (151032285, Qiagen, Hilden, Germany) to prevent remaining of unwanted cellular material. After centrifuged for 30 seconds the flow-through was removed. 500 µl 80% ethanol was added to the columns and centrifuged for two minutes. Again, the columns were put in a new collection tube and the samples were centrifuged with open lid for five minutes on maximum speed. A new and final 1.5ml tube (151030138, Qiagen, Hilden, Germany) was numbered corresponding the sample numbers. The RNeasy micro spin columns were placed in these tubes and 17µl RNase free water (151022581, Qiagen, Hilden, Germany) was pipetted directly on the membrane of the columns. After one minute incubation time the samples were centrifuged for one minute. The flow-through was collected and pipetted again directly on the membrane. For the last time the samples were centrifuged one minute before put on ice. This final flow-through contained the isolated RNA.

The concentration of RNA in the samples was measured by putting 1 µl of each sample on the Agilent 2100 Bioanalyser and RNA Nanochip kit (5067-1511, Agilent Technologies, Amstelveen, the Netherlands). To calculate the RNA concentration absorbance was measured at 260 nm. This concentration was used to calculate the needed amount for the next step, the cDNA synthesis.

Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands). Before synthesised, the needed amount per sample was calculated. This way, all the samples consisted of equal RNA amounts. Nuclease-free water was added till a total content of 15 µL per sample was reached. 4 µL of the iScript reaction mix and 1 µL of the iScript reverse transcriptase was added to each sample and mixed. Then, all the samples were incubated in the Dyad Disciple™ Peltier Thermal Cyclers (Bio-Rad, Veenendaal, the Netherlands) and the cDNA samples were stored in the freezer till qPCR was performed. In addition of the quantity measurement using the Nanochip, the quality of the isolated RNA was measured on cDNA samples by performing a Bio Analyser on randomly chosen samples per group.

qPCR

Primers	forward sequense	Reverse sequence	(bp)	(°C)
Col1	5'-GTGTGTACAGAACGGCCTCA-3'	5'-TCGCAAATCACGTCATCG-3'	109	61
Col2	5'-GCAGCAAGAGCAAGGAC-3'	5'-TTCTGAGAGCCCTCGGT-3'	150	60.5-65
ACAN	5'-GGACACTCCTTGCAATTTGAG-3'	5'-GTCATTCCACTCTCCCTTCTC-3'	110	61-62
MMP9	5'-CGCATGACATCTTCCAGTACCA-3'	5'-CCGAGAATTCACACGCCAGTA-3'	74	63
MMP13	5'-CTGAGGAAGACTTCCAGCTT-3'	5'-TTGGACCACTTGAGAGTTCG-3'	250	65
TIMP1	5'-GGCGTTATGAGATCAAGATGAC-3'	5'-ACCTGTGCAAGTATCCGC-3'	120	66
ADAMTS5	5'-CTACTGCACAGGGAAGAG-3'	5'-GAACCCATTCCACAAATGTC-3'	148	61
IL-6	5'-GAGCCCACCAGGAACGAAAGAGA-3	5'-CCGGGGTAGGGAAAGCAGTAGC-3'	123	65
PTGES1	5'-CCAGTATTGCCGGAGTGACCAG-3'	5'-AAACGAAGCCCAGGAACAGGA-3'	97	68
PTGES2	5'-GCTCTCAAGACCTACCTGG-3'	5'-AGTCACTTCCTTTCCCTGG-3'	98	60-62
BAX	5'-CCTTTTGCTTCAGGGTTTCA-3'	5'-CTCAGCTTCTTGGTGGATGC-3'	108	58-59
BCL2	5'-GGATGACTGAGTACCTGAACC-3'	5'-CGTACAGTTCCACAAAGGC-3'	80	61.5-63
CASP3	5'-CGGACTTCTTGTATGCTTACTC-3'	5'-CACAAAGTGACTGGATGAACC-3'	89	61
GAPDH	5'-TGTCCCCACCCCCAATGTATC-3'	5'-CTCCGATGCCTGCTTCACTACCTT-3'	100	58
HPRT	5'-AGCTTGCTGGTGAAAAGGAC-3'	5'-TTATAGTCAAGGGCATATCC-3'	104	56-58
SDHA	5'-GCCTTGGATCTCTTGATGGA-3'	5'-TTCTTGGCTCTTATGCGATG-3'	92	61
RPS19	5'-CCTTCCTCAAAAAGTCTGGG-3'	5'-GTTCTCATCGTAGGGAGCAAG-3'	95	61+63

Table 2 Primers of the canine target genes and Reference gene which were measured (Col1/Col2 = collagen type I/II; ACAN = aggrecan; MMP9/MMP13 = matrix metalloprotease 9/13; TIMP1 = tissue inhibitor of metalloproteinase 1; ADAMTS5 = a disintegrin and metalloproteinase with thrombospondin motifs 5; IL-6 = interleukin 6; PTGES1/PTGES2 = prostaglandin E synthase 1/2; BAX = BCL Associated X; BCL2 = B-cell lymphoma 2; CASP3 = Caspase 3) and reference genes (GAPDH = glyceraldehydes-3-phosphate dehydrogenase,; HPRT = Hypoxanthine-guanine phosphoribosyltransferase; SDHA = Succinate dehydrogenase complex, subunit A; RPS19= Ribosomal protein S19). Bp: the product size in base-pares, °C: the PCR temperature.

After synthesizing cDNA as described above, gene expression was quantified using quantified-Polymerase Chain Reaction (qPCR). The samples were first diluted 10 times. Then, a standard line was made by adding 9 µL of each sample in S1. The serial dilutions were made by each step diluting 4 times from S1 toward S7. Per target gene a master mix was made containing 264.5 µL iQT^{TM} SYBR green Supermix (Bio-Rad, Veenendaal, the Netherlands), 48.7 µL MQ and 2.1 µL of both 100 µM Forward and 100 µM reverse primers of the dog specific primers (see Table 2).

Of this super mix 6 µL was added into the wells of a 384-wells PCR plate. After this, 4 µL of 50 times diluted cDNA in each well was added. After centrifuged the plate was inserted in the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). The right temperature was entered according to the used primers. The samples were subjected to 40 amplification cycles with fluorescence detection after each cycle. The cycle in which the fluorescence reached the threshold value determined the amount of starting material and thereby the degree to which the gene of interest is activated under the conditions examined. Four reference genes were quantified to normalize the target genes expression(GAPDH, HPRT, SDHA and RPS19)

Histology

Safranin-O / Fast Green staining

Paraffin embedded sections of samples collected at day 28 were made on which the staining would be performed for visualizing the proteoglycan content(see Figure 6). First, the slides were deparaffinised with xylene (two times five minutes) and rehydrated by a series of decreasing dilutions of ethanol (96 %, 80 %, 70 %, 60 %; five minutes each) and ends with five minutes milliQ rinsing step. The slides were placed in Citric acid for ten minutes while being placed on the rocking machine. A five minute washing step under running distilled tap water followed. The sections were subjected to Weigerts's Hematoxylin (640505, Klinipath B.V., Duiven, The Netherlands) for five minutes. The washing step was repeated for ten minutes before the sections were counterstained with 0.4% Fast Green (F7252, Sigma-Adrich, Saint Louis, USA) for four minutes. The sections were then rinsed in 1% Acetic Acid, two times five minutes, untill colour no longer ran. After draining the section they were placed in 0.125% Safranin-O (58884, Sigma-Aldrich, Saint Louis, USA) staining for five minutes. The sections were then dehydrated in 96% ethanol (two times three minutes), 100 % ethanol (two minutes) and xylene (two times five minutes) and mounted (Vectamount, H5000, Vector Laboratories, Burlingame, USA).

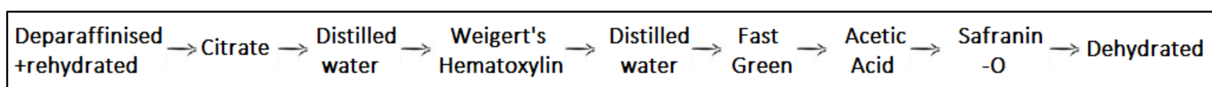

Figure 6 Schematic overview of the Safranin-O/ Fast Green staining

Biochemical analyses

The glycosaminoglycan(GAG) content of the pellets collected at day 28 and of the medium collected at each medium change were measured using dimethylene blue dye binding (DMMB)assay.

To determine the GAG content of the pellets the pellets were first digested overnight in a papin digestion solution(pH 6, 200mM H2NaPO4 * 2 H2O)(21254, Boom B.V. Meppel, the Netherlands) 10mM EDTA(100944, Merck Millipore, Amsterdam, the Netherlands.), 10mM cysteine HCL(C7880, Sigma-Aldrich, Saint Louis, USA) and 10 mM papain (P3125, Sigma-Aldrich, Saint Louis, USA). After this, the DMMBassay was performed, using an earlier made staining solution (16 mg DMMB added to 5 ml of 100% ethanol, incubated while on a roller bench for 2-16 hours. 2.37 g NaCl and 3.04 g Glycine added to 1 L distilled water. When the pH is set on 3.0 the DMMB was added to this solution.) A standard line was made of chondroitin sulphate C(0.5mg/ml) diluted 1:50 in PBS-EDTA. 100 µL of this standard line was pipetted in duplicate in a 96-wells plate. Directly after 100 µL of the diluted samples were pipetted in the wells 200 µL of the DMMB staining solution was added. The plate was placed in a microplate reader(Tecan) were the extinction was measured at 525 and 595 nm. These results were calculated to the total GAG content in each sample.

The DNA contents of the pellets collected at day 28 were measured using he Qubit ds DNA High Sensitivity Assay Kit (Q32851, Invitrogen , Paiseley, UK). First the working solution was made by, depending on the sample size, adding the Qubit Reagent to the Qubit Buffer in a 1:200 ratio. The standard was created by adding 10 µL of Standards (S1 and S2, delivered by the Qubit kit) to 190 µL of the working solution. Than 5 µL of each sample was added to 195 µL of the working solution. After an incubation time of 2-3 minutes (at room temperature) the DNA content was measured in the Qubit 2.0 Fluorometer. Results were shown as DNA in ng/ml.

Statistical analyses

For the statistical analysis SPSS Statistics 22 was used. Using the Shapiro Wilks test, data were divided in normal and a non-normal distributed data. Normal distributed data were further analysed using the one-way ANOVA test. All non-normal distributed data were analysed using the Kruskall Wallis test to verify if there were significant differences within the data. The genes that had a significant outcome ($p<0.05$) were further analysed using the Mann-Whitney U test to specify between which conditions a statistical difference was seen. To correct for multiple comparisons a post-hoc test was performed on all significant outcomes using the Benjamini and Hochberg method. Only the significant differences that showed two-fold changes were seen as biologically relevant.

Results

Gene expression

In Figure 7 the expression of genes associated with extracellular matrix production measured in pellets collected at day 7 is shown. TNF-α treated pellets, with and without celecoxib, showed an increase in expression of Col1 compared to the control group (Figure 7A, $p<0.01$). Both *Col2* and *ACAN* showed a decrease in expression of pellets treated with TNF-α with or without celecoxib (Figure 7B and D, $p<0.01$). Expression of *ACAN* shows a significant decrease in the celecoxib treated pellets compared to the pellets treated with TNF-α(Figure 7 D, $p<0.01$). The ratio of Col2 and Col1 was calculated to compare the relative changes of expression and shows in the treated pellets a significant decrease compared to the control, meaning that in that *Col1* more is expressed relative to *Col2*(Figure 7C, $p<0.01$).

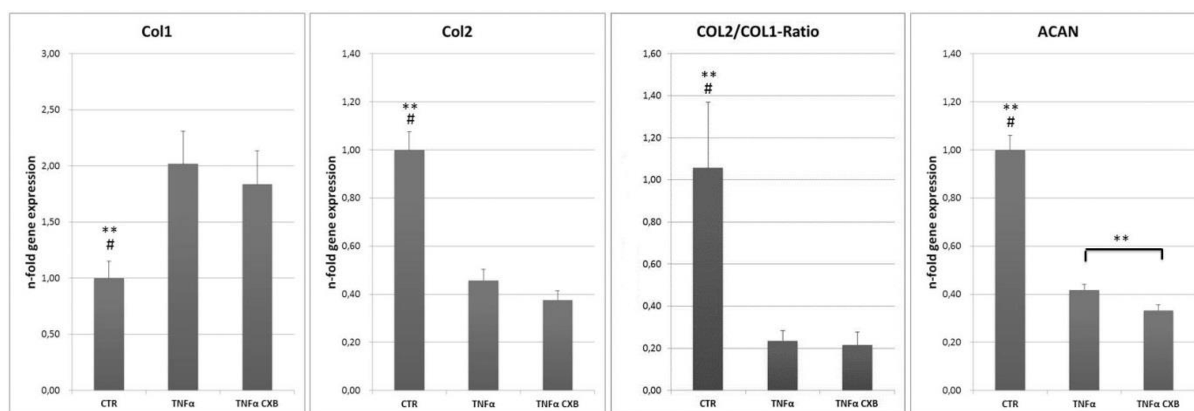

Figure 7 Relative gene expression of genes associated with extracellular matrix production. Measured in Nucleus pulposus cells cultured in pellets collected at day 7. **A.** Expression of collagen type 1 (*Col1*); **B.** Expression of collagen type 2 (*Col2*); **C.** *Col2/Col1*-ratio; **D.** Expression of *Aggrecan (ACAN)*. Data are expressed in n-fold (relative) gene expression and are shown with standard deviation bars, except for the *COL2/COL1*-ratio. # indicates significance difference between all other conditions, ** = $p<0.01$. *CTR*= control; *TNFα*= 10 ng/ml Tumor Necrose factor (TNFα) was added to the pellets to induce inflammation; *TNFα CXB*= bolus of 0.013 mg/ml celecoxib(CXB) was added to the pellets induced with TNFα.

Figure 8 shows the expression of genes associated with extracellular matrix remodeling/degradation. The expression of the catabolic genes are upregulated in pellets treated with TNF-α and CXB compared to the control group(Fig A,B and C). For MMP 13, specific degrading type 2 collagen the significant difference varies. Between the CTR and the TNF-α treaded pellets an upregulation is seen with $p<0.01$. The significant difference between CTR and celecoxib pellets is with $p<0.05$.

The relative expression of genes associated with inflammation is showed in Figure 9. Only for *IL6* , a cytokine that regulates the balance of matrix proteins, a significant decrease in the expression of control pellets compared to TNF-α pellets. An upregulation is seen by an increase of expression in celecoxib pellets compared to TNFα pellets(Fig. 9C, $p<0.05$).

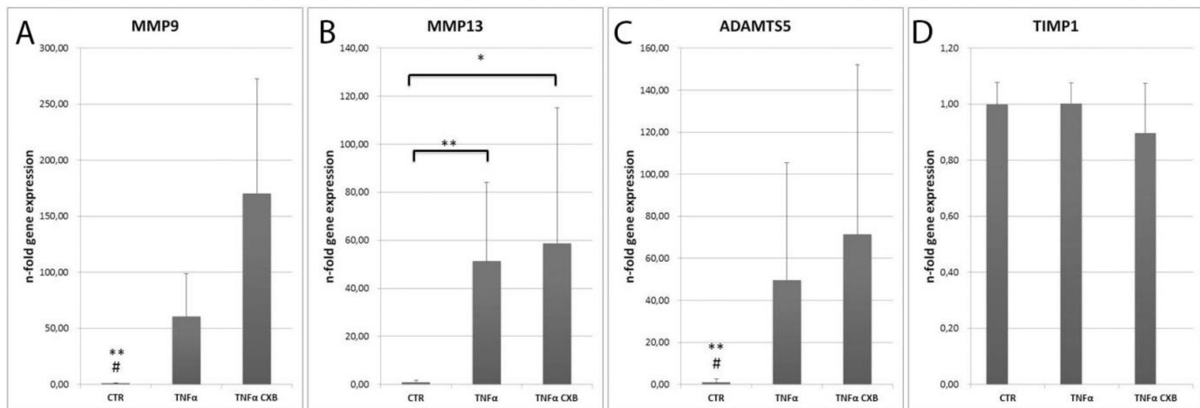

Figure 8 Relative gene expression of genes associated with matrix remodeling/degradation. Measured in Nucleus pulposus cells cultured in pellets collected at day 7. **A.** Expression of matrix metalloprotease 9 (*MMP9*); **B.** Expression of matrix metalloprotease 13 (*MMP13*); **C.** Expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*); **D.** Expression of tissue inhibitor of metalloproteinase 1 (*TIMP1*). Data are expressed in n-fold (relative) gene expression and are shown with standard deviation bars. # indicates significance difference(p<0.05) between all other conditions, * = p<0.05, ** = p<0.01. *CTR*= control; *TN-α*= 10 ng/ml Tumor Necrose factor (TNF-α) was added to the pellets to induce inflammation; *TNFα CXB*= bolus of 0.013 mg/ml celecoxib(CXB) was added to the pellets induced with TNF-α.

In Figure 10 relative gene expression of the genes associated with apoptosis are shown. For the anti-apoptotic gene *BCL2* a significant decrease in expression is shown in TNF-α and CXB pellets compared to the control (fig. 10B, p<0.01). The ratio of *BAX* and *BCL2* was calculated to compare the relative changes of expression between the apoptotic gene *BAX* and the anti-apoptotic gene *BCL2*. It is shown that in pellets treated with TNF-α shown a significant increase, meaning there is relative more expression of the apoptotic gene Bax compared to BCL2 (Fig 10C, p<0.01). In cxb treated pellets this increase is present but less dominant shown by a significance of p<0.05.

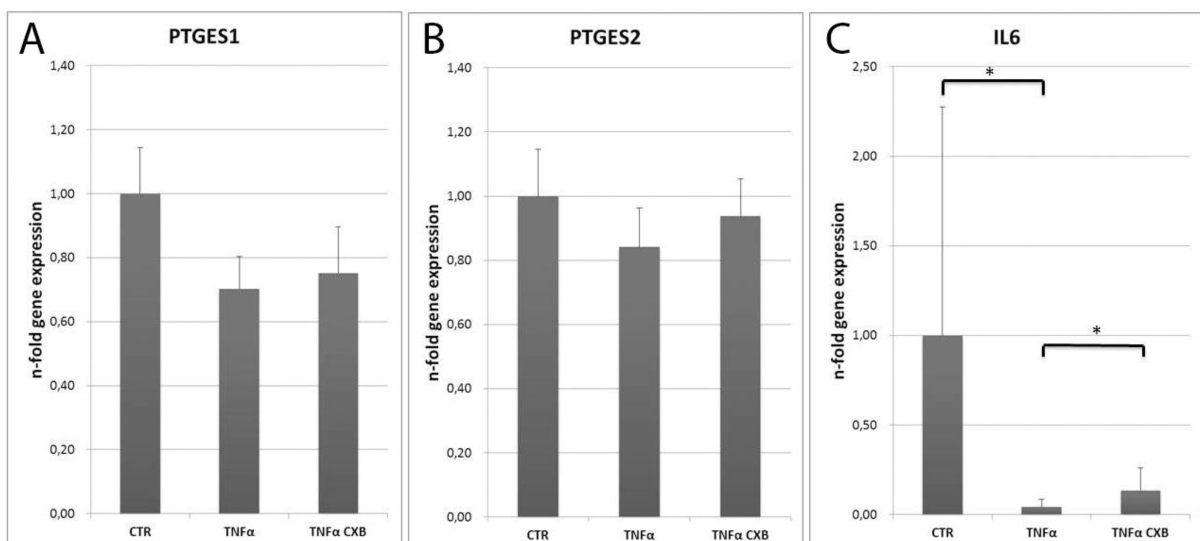

Figure 9 Relative gene expression of genes associated with inflammation. Measured in Nucleus pulposus cells cultured in pellets collected at day 7. **A.** Expression of prostaglandin E synthase 1 (*PTGES1*); **B.** Expression of prostaglandin E synthase 2 (*PTGES2*); **C.**Epression of Interleukin 6 (*IL6*). Data are expressed in n-fold (relative) gene expression, and are shown with standard deviation bars. # indicates significance difference (p<0.05) between all other conditions, * = p<0.05 *CTR*= control; *TNFα*= 10 ng/ml Tumor Necrose factor (TNF-α) was added to the pellets to induce inflammation; *TNFα CXB*= bolus of 0.013 mg/ml celecoxib(CXB) was added to the pellets induced with TNF-α.

Quality of isolated RNA was measured by performing a Bio Analyser randomly chosen samples per group. The RNA Integrity Number (RIN) varied between 10 and 7.4.

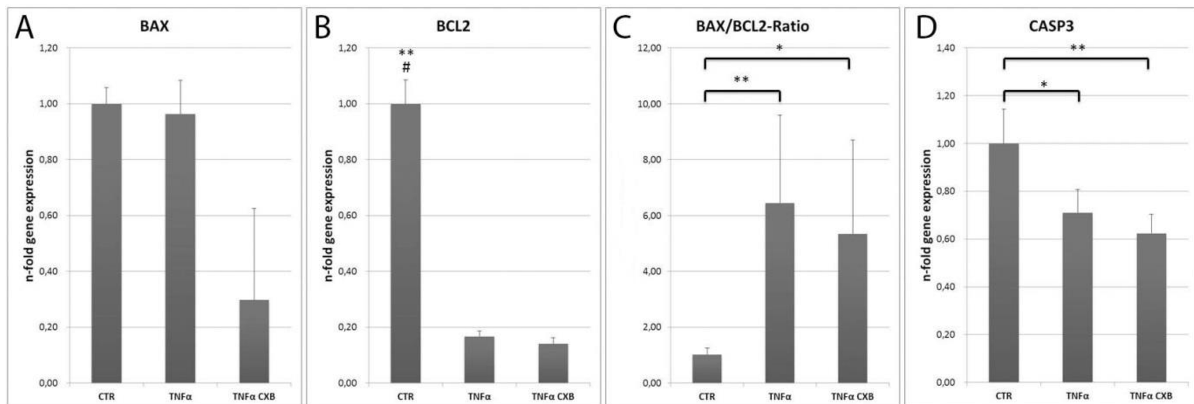

Figure 10 Relative gene expression of genes associated with apoptosis. Measured in Nucleus pulposus cells cultured in pellets collected at day 7. **A.** Expression of BCL Associated X (*BAX*); **B.** Expression of B-cell lymphoma 2 (*BCL2*); **C.***BAX/BCL2*-ratio; **D.** Expression of Caspase 3 (*CASP3*). Data are expressed in n-fold (relative) gene expression and are shown with standard deviation bars, except the *BAX/BCL2*-ratio. # indicates significance difference ($p<0.05$) between all other conditions, * = $p<0.05$, ** = $p<0.01$. *CTR*= control; *TNFα*= 10 ng/ml Tumor Necrose factor (TNF-α) was added to the pellets to induce inflammation; *TNFα CXB*= bolus of 0.013 mg/ml celecoxib(CXB) was added to the pellets induced with TNF-α.

Safranin-O / Fast Green staining

Safranin O/fast green is a staining method used to visualize proteoglycans present in tissue. Nuclei will dye black/dark blue. Glycosaminoglycan will dye red. The Safranin O staining showed a minimal decrease of GAG deposition in the matrix of both TNF and CXB treated pellets compared to the control group (**Figure 11**).

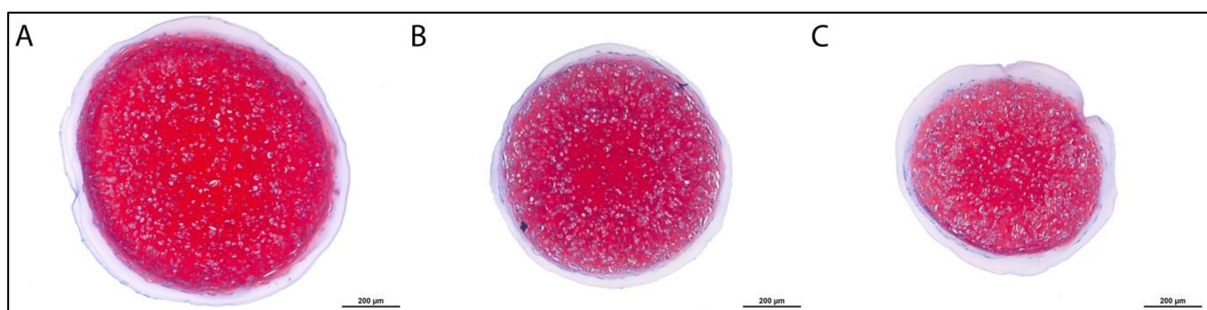

Figure 11. Histological images of nucleus pulposus cells cultured in pellets for 28 days stained with Safranin-O/Fast Green A. Control condition; **B.** TNF-α condition and **C.** TNF-α bolus celecoxib treatment.

Biochemical analyses

Pellets

Figure 12A Shows the Glycosaminoglycan (GAG) content of the pellets collected at day 28. The pellets treated with TNF-α showed a significant decrease in GAG content compared to the control pellets and the pellets treated with CXB. Compared with the control group the GAG content was significantly lower in the pellets treated with celecoxib.

DNA content of the pellets is shown in Figure 12B, representing the amount of viable cells. It was shown that the DNA content significantly decreased in the pellets treated with TNF-α and pellets treated with CXB compared to the control group.

The ratio GAG/DNA of the pellets was calculated to define the GAG content per cell and is shown in Figure 12C. It was shown that celecoxib treated pellets produced significantly more GAG per cell compared to the control group.

Medium

Figure 13 shows the GAG content measured in the collected medium before each medium change. Figure 13A shows how the GAG content is changing over time. Figure 13B shows the accumulate amount of GAG content in medium during 28 days, including the GAG content of the pellets. This is an indication of the total amount of GAG produced by the pellets. No statistical analysis could be performed while the sample size was too small.

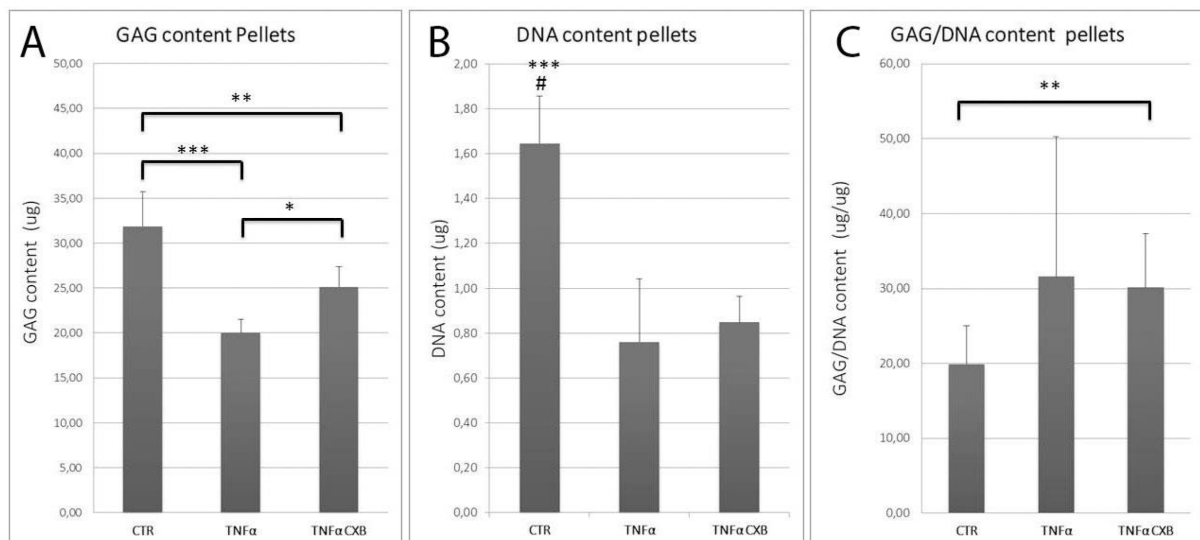

Figure 12 Glycosaminoglycan (GAG), DNA, and GAG/DNA content in cultured nucleus pulposus cells (NPCs) pellets at day 28. Data are expressed as mean and are shown with standard deviation bars. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. *CTR= (negative) Control; TNFα= Tumor Necrose factor α (positive control) ; TNFα CXB= TNFα and celecoxib bolus.*

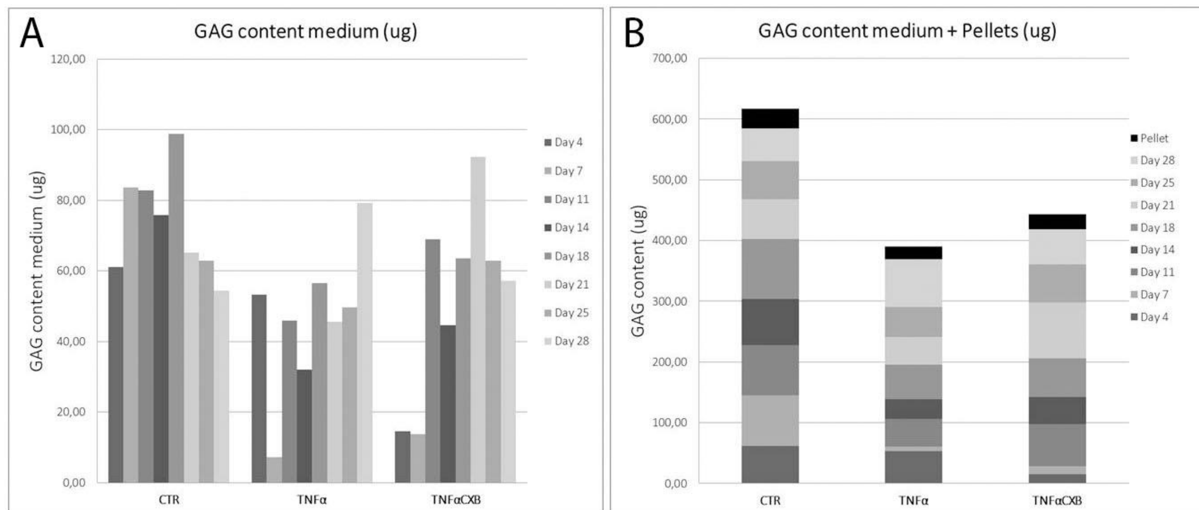

Figure 13 Glycosaminoglycan (GAG) release in culture medium. A. GAG release in medium at day 4, 7, 11 ,14, 18, 21, 25 and 28 **B.** Cumulative GAG release in medium and pellets. *CTR*= (negative) Control; *TNFα*= Tumor Necrose factor α (positive control) ; *TNFα CXB*= TNFα and celecoxib bolus.

Discussion inflammatory model

This pilot study was performed to create an applicable model for research of the sustained anti-inflammatory effect of celecoxib loaded hydrogels.

Gene expression of anabolic genes showed an alteration in content of matrix molecules after inflammation was induced by tumor necrosis factor α (TNF-α). As expected the contents changed from a relative high *Col2* and *Acan* expression to a significantly lower expression in pellets treated with TNF-α. This is in line with earlier published literature.[15] The relative low *Col1* expression significantly increased after inflammatory induction (Figure 7). *In vivo* these changes are described as the reason for turnover to a more fibrotic/rigid consistency of the nucleus pulposus in degenerated IVD.[3] The anti-inflammatory effect of celecoxib on the expression of the anabolic genes was unclear. There were no significant improvements of the gene expression of *Col1*, *Col2* and *Acan* of the pellets treated with celecoxib compared to the NP pellets treated with TNF-α. On the other hand the expression of *Acan* showed even a significant decrease in the pellets treated with CXB. This decrease is in contrast to the expectations that CXB would reduce the inflammatory effect of TNF-α and thereby stimulated the Acan and Col2 expression. According to the authors knowledge no studies are performed to evaluate the effect of celecoxib on nucleus pulposus cells. Effects of another NSAID (diclofenac) have been tested before on nucleus pulposus cells.[16] In this study bovine nucleus pulposus cells were treated with diclofenac and showed an increase of Aggrecan expression. A reasonable explanation that celecoxib did not show this expected anti-inflammatory effect may be an inadequate used dosage or an different pathway of the effect of celecoxib compared to diclofenac. Although, diclofenac and celecoxib showed similar anti-inflammatory effects on management of rheumatoid arthritis.[17] Except for the side effects, which were severe when used diclofenac, due to the non-selective COX inhibition.

The PCR results showed the changes in gene expression of the matrix metalloproteinases (*MMP9* and *MMP13*) and *ADAMTS5* as well as their regulator the tissue inhibitors of matrix metalloproteinases (*TIMP1*). For both *MMP's* and for *ADAMST5* a significant increase of

expression was shown in pellets treated with TNF-α compared to the CTR. In contrast to these dramatic increases of gene expression, there were no changes shown in the expression of *TIMP1*, the endogenous inhibitor of MMPs. This same phenomenon is published in a similar research only performing PCR after 48 hours.[15] Possibly, these results can be explained by previous published effects of Collagen type 2 fragments on the NP cells, stimulating MMP expression without any effect on TIMP expression.[18] A significant increase was shown of the expression of all catabolic genes in pellets treated with celecoxib compared to the control group. These results are not in line with the expectations. Because the anti-inflammatory effect of celecoxib should prevent breakdown of the matrix-content and these data did not show that. It is a possibility that this is due to an inadequate used dosage of celecoxib. Another reason can be that these negative effects are due to the presence of ethanol in the CXB bolus treatment, which was used to dilute CXB to the wanted concentration. Ethanol was also used, although in higher concentration, to induce an degenerated intervertebral disc model.[19] For future research this should be taken into account by creating an extra condition of nucleus pulposus cells which are only treated with the same concentration ethanol as used to dilute CXB.

PTGES1 and PTGES2 showed no significant changes in expression after the pellets were treated with both TNF or CXB. This is in contrast with the expectation because according to known literature TNF would stimulate an increased expression of prostaglandin E synthases (PTGES1 or PGES1). Pro-inflammatory stimuli will increase the expression of this enzyme both *in vivo* and *in vitro*.[10] These same changes have been described of the expression of IL-6 as a result of pro-inflammatory stimuli. The present study showed only a significant decrease in expression of IL-6 after TNF-α stimulation. It is investigated in arthritis that IL-6 has both an anti-inflammatory effect as a pro-inflammatory effect.[20] This may suggest that the pro-inflammatory effect of TNF-α is predominant over the anti-inflammatory or pro-inflammatory effect of IL-6 and therefor the expression of IL-6 is downregulated. [21]

For the anti-apoptotic gene BCL2 a significant decrease was showed in both the TNF- α treated pellets and the CXB treated pellets compared to the control group. Overall, showed in the BAX/BCL2-ratio, the pro-apoptotic gene expression was predominant over the anti-apoptotic. This is an expected outcome after inducing inflammation and it is confirmed by the results of the DMMB assay, showing a significant decrease of DNA content in pellets after 28 days treatment with TNF-α. For both PCR and DMMB analysis treatment with celecoxib showed no improvement on the apoptotic expression in the pellets. Although looking at GAG production relative to DNA content a significant improvement was shown in pellets treated with CXB compared to the control group. Meaning that de viable cells in the pellets produced a higher amount of GAGs after treatment with celecoxib compared to the control.
Unfortunately our histological data did not comprehend agree with previous mentioned data of the PCR analysis. Where PCR showed the use of TNF-α as a reliable induction for inflammation, there are no optical inflammatory effects shown in the pellets stained with Safranin O/fast green. This might be due to the different collecting time points. Whereas PCR was performed on samples collected at day 7, histological staining was performed on samples collected at day 28.

Overall this present research showed an inflammatory response due to Tumor Necrosis Factor-α in nucleus pulposus cells, which simulated a realistic environment compared to the

degeneration seen in intervertebral discs for the first 7 culture days. This suggests that TNF-α is a reliable candidate to induce an *in vitro* environment comparable for intervertebral disc degeneration *in vivo*. It is recommended to further investigate the influence of the length of culturing on the inflammatory effect of TNF-α. Minimal effect of celecoxib was seen, since no clear anti-inflammatory effect of celecoxib on the nucleus pulposus cells was shown. A possible reason for this is an inadequate used doses of celecoxib. We used a dosage of 10^-7M celecoxib. Another study used 1 mg of CXB-loaded per ml of hydrogel leading to a concentration of 10µM CXB per culture medium. Although this study only showed a limited inhibition of PGE2 in this model with mild IVD degeneration after controlled release of CXB.[22]

In the next study it should be taken into account to investigate the adequate effective doses of celecoxib. Due to the possible low concentration of celecoxib in this present study, in the next hydrogel study a range of increasing concentrations of celecoxib should be examined. Another possible reason for the absence of the anti-inflammatory effect of celecoxib is that the collecting time point of samples for PCR measurement was inefficient. Various studies measure the gene expression by performing PCR more quickly after starting the experiment.[16,18,23] This should be taken into account in the next hydrogel study.

Hydrogel study

Study design

After this pilot study a following study was performed to examine the sustained anti-inflammatory effect of a celecoxib loaded thermo-gel *in vitro* on the NP cells in an inflamed environment, simulating IVD degeneration. The design of the study is shown in Figure 14.

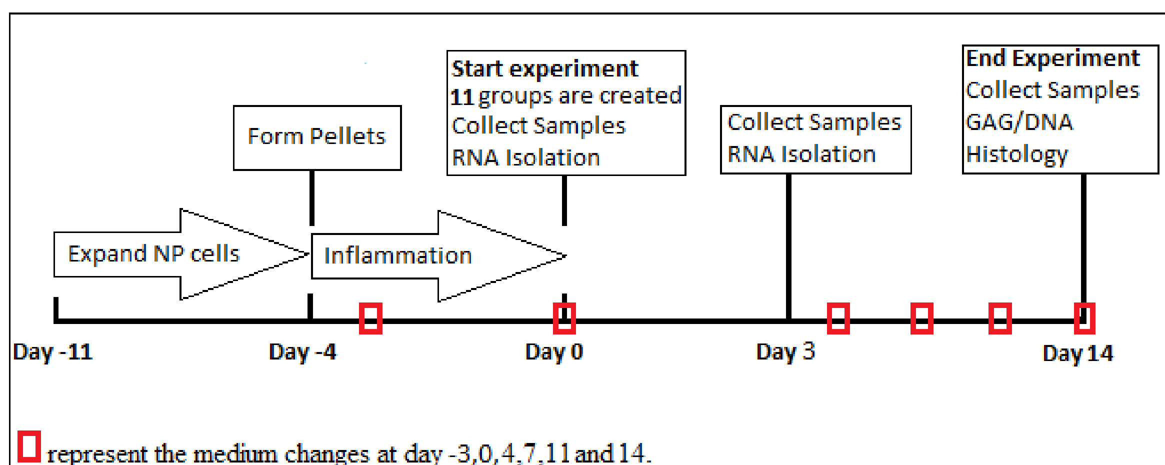

Figure 14 Design of study plotted over time. During the first seven days the pooled nucleus pulposus cells were expanded. After pellets were formed inflammation was induced in all conditions, except the control group, by adding Tumor Necrosis Factor α (TNF-α). At day 0 the experiment started and 11 different condition groups were created (see Table 3). At day 3 samples were collected in triplicate for gene expression measurements. At day 14 the experiment ended and the samples were used for biochemical analysis and histological staining.

After expanding nucleus pulposus cells for seven days pellets were formed. These pellets were cultured in basic chondrogenic medium and 5 ng/ml TGF- β was added to support the cells to remain vital. 10 ng/ml TNF-a was added to all the pellets except in the control group. At day 0, 11 different condition groups were created by adding different treatments to the culture medium each medium change (see Table 3). Except for the hydrogel, these same gels were replaced in the same wells after medium change.
To determine the optimal dose of celecoxib, three different doses of CXB bolus treatments were evaluated; 10^16M, 10^-5 M and 10^-4M. To achieve these increasing concentrations, celecoxib was diluted using two different amounts of ethanol. CXB10^-6 contained a lowest concentration of 0.04% ethanol and CXB10^-5 M and CXB10^-4M were diluted in 0.4% ethanol. To differentiate if an effect was due to the celecoxib or the present ethanol, two different groups were created in with the NP pellets were treated only with 0.04% and 0.4% ethanol. To control if there was a difference in gene expression between the start of the experiment and the three days later, planned RNA sample collection. Samples of day 0 were measured as well for the same genes.

Groups	Chondrogenic medium + TGF- β	TNFα	Loaded Thermo-gel	Unloaded Thermo-gel	Bolus CXB	EtOH
1. D0 Control	+					
2. D0 TNF	+	+				
3. Control	+					
4. TNF-α	+	+				
5. Loaded	+	+	+			
6. Unloaded	+	+		+		
7. CXB 10^-6M	+	+			+	+
8. CXB 10^-5M	+	+			+	+
9. CXB 10^-4M	+	+			+	+
10. EtOH 10^-6M	+	+				+
11. EtOH 10^-5M	+	+				+

Table 3: Additives at day 0 per group. *Group 1*: Pellets cultured in medium with 5 ng/ml TGF- β and collected at day 0. *Group 2:* 10 ng/ml TNF-α was added to control the inflammatory effect and pellets were collected at day 0. *Group 3*: Pellets cultured in medium with 5 ng/ml TGF- β. *Group 4:* 10 ng/ml TNF-α was added to control the inflammatory effect. *Group 5:* 10 ng/ml TNF-α and celecoxib (CXB)-loaded hydroo-gel was added to evaluate the sustained effect of this drug delivery system. The doses of CXB processed in the hydrogel was similar to the 10^-5M bolus added in group 8. *Group 6:* 10 ng/ml TNFα in combination with unloaded thermo-gel. *Group 7:* 10^-6M celecoxib was added. *Group 8:* 10^-5M celecoxib was added. *Group 9:* 10^-4M celecoxib was added. *Group 10:* EtOH 10^-6M was added containing 0.04% ethanol. *Group 11*: EtOH 10^-5M was added containing 0.4% ethanol.

Results

Gene expression

Effect of TNF-α on the gene expression of nucleus pulposus cells
Gene expression of the pellets treated with TNF-α was compared to the pellets of the control group. This way the inflammatory effect could be distinguished. For the anabolic genes *Col2* and *ACAN* there was a decrease in expression of the nucleus pulposus cells treated with TNF-α. Only for the *Col2*/*Col1* ratio that was measured, to compare the relative changes of expression, a significant decrease was shown. This means that in the TNF-α treated pellets, the expression of *Col1* is dominant over *Col2*. While in the TGF-β pellets, *Col2* was dominant. No significant changes were shown in the catabolic gene expression, except for the tissue inhibitor of metalloproteinase 1 (*TIMP1*), which showed an increase when treated with TNF-α (see Figure 16D, $p<0.05$). The expression of inflammation mediator *IL-6* was upregulated in the pellets treated with TNF-α (see Figure 17C, $p<0.05$). On apoptotic gene expression there were some significant differences between samples collected at day 0 and collected at day 3 (see Figure 18). Overall, compared to TGF-β pellets, an increase of pro-apoptotic gene expression was shown (*BCL2*) in combination with a decrease of anti-apoptotic gene expression (*CASP3*).

Effect of celecoxib-loaded hydrogel on the gene expression of nucleus pulposus cells
The gene expression of the pellets treated with the loaded hydrogel was compared to the pellets treated with TNF-α and compared to CXB bolus treatment of 10^-5M, which was the same concentration as added in the hydrogel. This way the anti-inflammatory effect could be seen and a comparison could be made of the amount of CXB that was released by the hydrogel. Loaded hydrogel showed a decrease of gene expression of *Col1* compared to both TNF-α and the corresponding CXB concentration. On gene expression of *Col2* and *Acan* the loaded gel showed a downregulation compared to TNF-α. For the catabolic genes, loaded hydro-gel showed a downregulation of the expression of MMP9, compared to TNF-α and CXB 10^-5M. For ADAMTS5 an upregulation was showed in the pellets treated with TNF-α (see Figure 16, $p<0.05$). The anti-apoptotic gene *BCL2* showed an increase of expression compared to the TNF-α group.

Effect of unloaded hydrogel on the gene expression of nucleus pulposus cells
To control the effect of the unloaded hydrogel in order to investigate the tolerability of the administration on nucleus pulposus cells, gene expression was compared to the pellets treated with TNF-α and compared to the loaded hydro-gel. The unloaded gel showed an effect on gene expression of *Col2*, *Acan*, *ADAMST5* and *PTGES2*, compared to the loaded hydro-gel. For the gene expression of *MMP13* and *TIMP1* the unloaded gel showed a decrease compared to the TNF-α treated pellets. On *IL-6* and *BCL2* an increase of gene expression compared to TNF-α was shown.

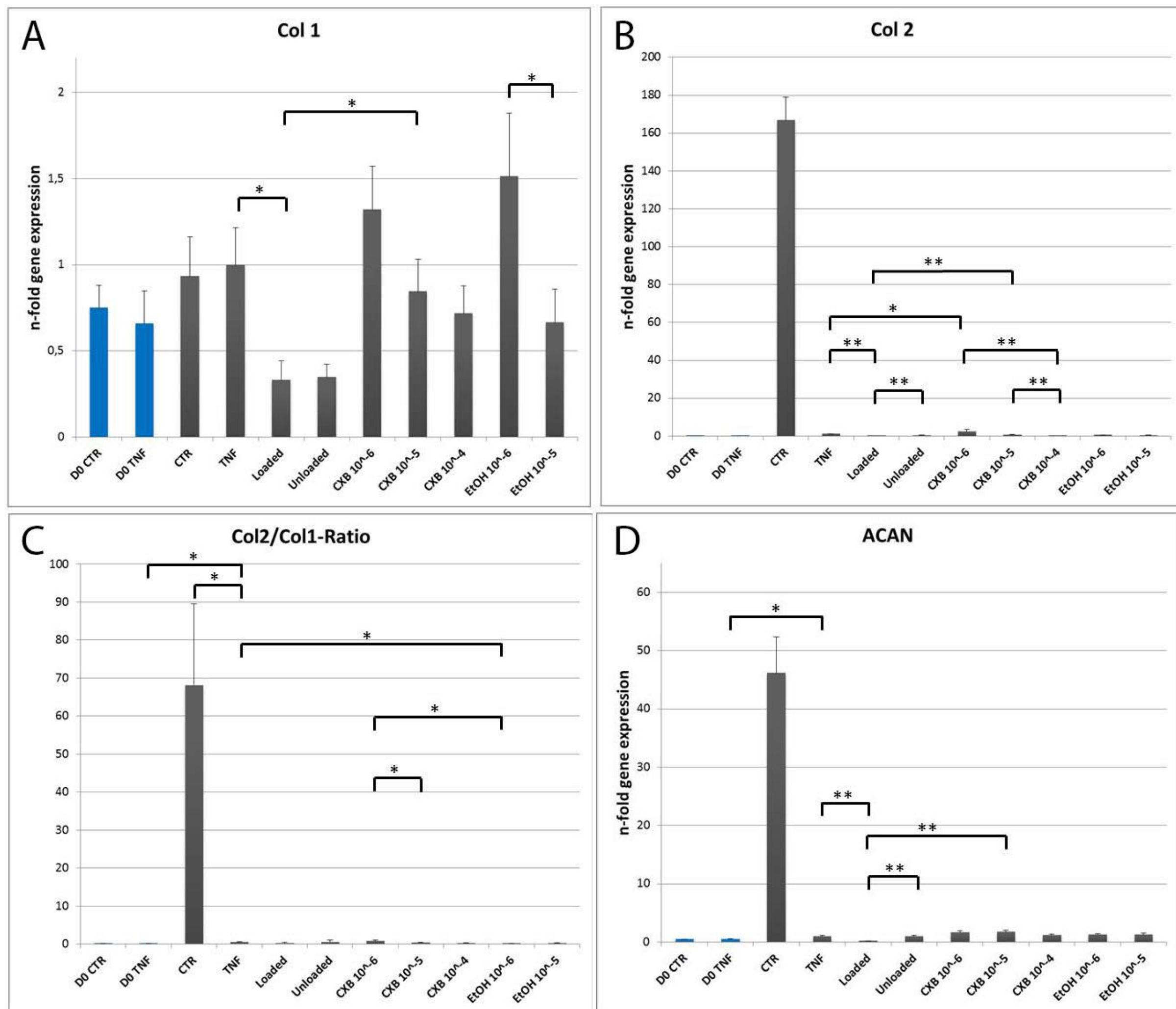

Figure 15 Relative gene expression of genes associated with extracellular matrix production. Measured in nucleus pulposus cells cultured in pellets collected at day 0 (indicated by the blue bars) and day 3 (indicated by the grey bars). **A.** Expression of collagen type 1 (*Col1*); **B.** Expression of collagen type 2 (*Col2*); **C.** *Col2/Col1*-ratio; **D.** Expression of *Aggrecan (ACAN)*. Data are expressed in n-fold gene expression, except for the *COL2/COL1*-ratio and are shown with standard deviation bars. *Indicates significant defferences with $p<0.05$ and ** = $p<0.01$. *D0 CTR*= positive control samples collected at day 0; *D0 TNF*= Negative control samples treated with 10 ng/ml Tumor Necrose factor (TNF-α) collected at *day 0; CTR*= positive control samples collected at day 4; *TNF*= negative control pellets treated 10 ng/ml TNFα; *Loaded*= 10 ng/ml TNF-α and celecoxib (CXB)-loaded hydro-gel were added to the pellets; *Unloaded*= 10 ng/ml TNF-α and unloaded hydro-gel were added to the pellets; *CXB10^-6*= 10 ng/ml TNF-α and bolus of celecoxib (CXB) in a 10^-6 concentration was added to the pellets; *CXB10^-5*= 10 ng/ml TNF-α and bolus of CXB in a 10^-5 concentration was added to the pellets; *CXB10^-4*= 10 ng/ml TNF-α and a bolus of CXB in a 10^-4 concentration was added to the pellets; EtOH10^-6=10 ng/ml TNF-α and a bolus of ethanol(EtOH) in a 10^-6 concentration was added to the pellets; EtOH10^-5=10 ng/ml TNF-α and a bolus of EtOH in a 10^-5 concentration was added to the pellets.

The effect of the increasing doses of celecoxib bolus treatment on gene expression of nucleus pulposus cells

Because the release of celecoxib by the loaded hydro-gel is unknown, concentrations of CXB bolus treatment can function as a reference of the range of release, by comparing which CXB concentration is most similar to the effect of the loaded hydro-gel. The highest used concentration CXB is higher than the total concentration processed in the loaded hydro-gel. This can serve to differentiate if the lack of an anti-inflammatory effect of the loaded hydro-gel is due to a too low release of CXB or due to a non-functional loaded hydro-gel.

A dose dependent response was shown in gene expression of COL2, ADAMTS5,IL-6 and CASP3. For the inflammatory genes, the highest concentration (CXB 10^-4) showed the most upregulation of gene expression compared to TNF-α and the other two lower concentrations (see Figure 17). This same effect is also shown in gene expression of pro-apoptotic CASP3 (Figure 18D, $p<0.001$).

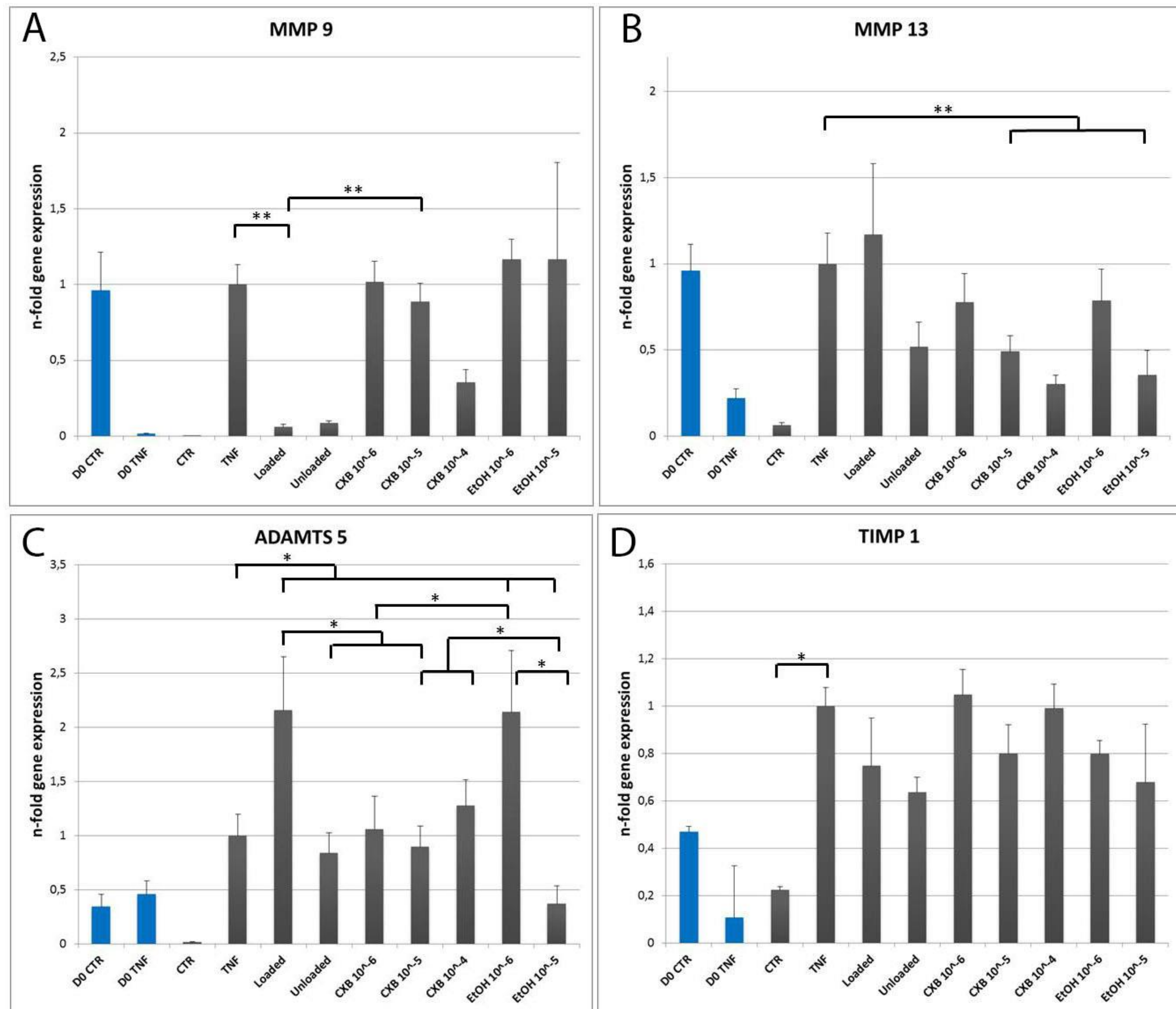

Figure 16 Relative gene expression of genes associated with matrix remodeling/degradation. Measured in nucleus pulposus cells cultured in pellets collected at day 0 (indicated by the blue bars) and day 3 (indicated by the grey bars).**A.** Expression of matrix metalloprotease 9 (*MMP9*); **B.** Expression of matrix metalloprotease 13 (*MMP13*); **C.** Expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*); **D.** Expression of tissue inhibitor of metalloproteinase 1 (*TIMP1*). Data are expressed in n-fold gene expression and are shown with standard deviation bars. *Indicates significant differences with $p<0.05$; ** = $p<0.01$.

Effect of ethanol on gene expression of nucleus pulposus cells

To control if the presence of ethanol in the CXB bolus concentrations had any effect, gene expression of the pellets treated with the two concentrations of ethanol were measured. Effects were compared to the corresponding CXB bolus dose and compared with the pellets treated with TNF-α. On gene expression of the anabolic genes ethanol showed no effects. Expression of MMP13 is decreased by the highest concentration ethanol compared to the TNF-α. For ADAMST5 the lowest concentration showed an increase in expression while the highest concentration a significant decrease compared to TNF-α. A dose dependent reaction is also seen in PTGES1, PTGES2, IL-6, BAX and CASP3 (see Figure 17 and Figure 18).

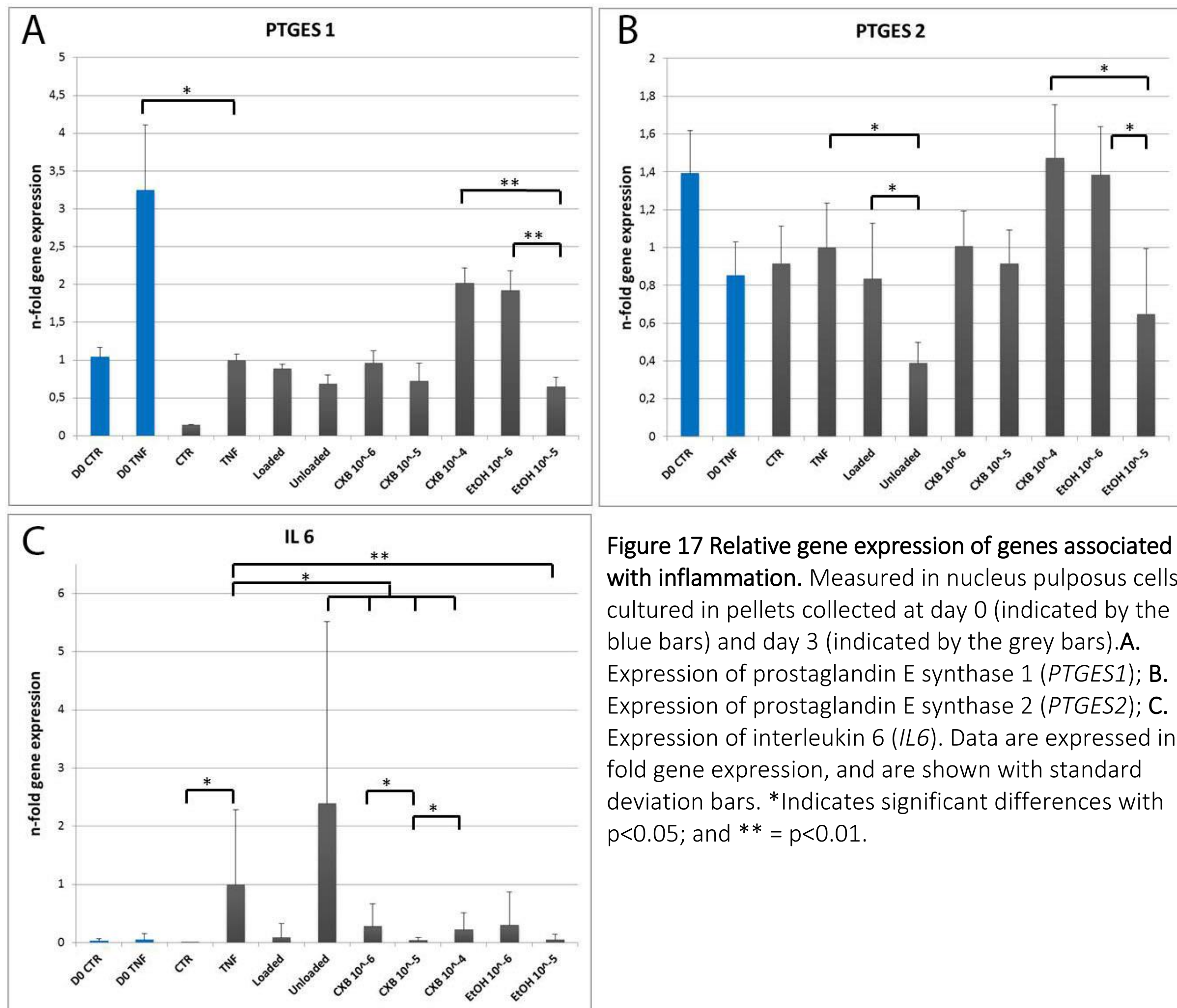

Figure 17 Relative gene expression of genes associated with inflammation. Measured in nucleus pulposus cells cultured in pellets collected at day 0 (indicated by the blue bars) and day 3 (indicated by the grey bars).**A.** Expression of prostaglandin E synthase 1 (*PTGES1*); **B.** Expression of prostaglandin E synthase 2 (*PTGES2*); **C.** Expression of interleukin 6 (*IL6*). Data are expressed in n-fold gene expression, and are shown with standard deviation bars. *Indicates significant differences with $p<0.05$; and ** = $p<0.01$.

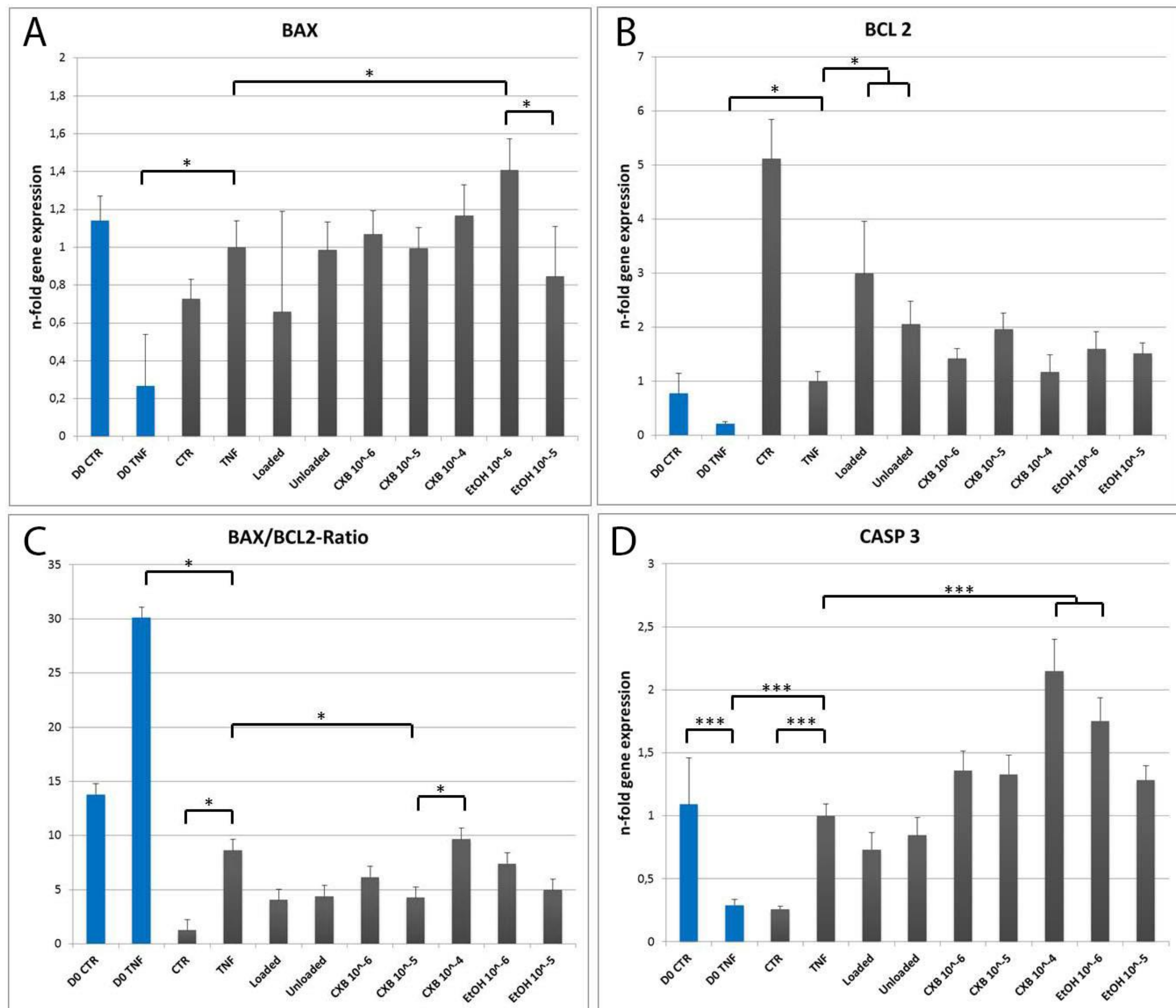

Figure 18 Relative gene expression of genes associated with apoptosis. Measured in nucleus pulposus cells cultured in pellets collected at day 0 (indicated by the blue bars) and day 3 (indicated by the grey bars).**A.** Expression of BCL Associated X (*BAX*); **B.** Expression of B-cell lymphoma 2 (*BCL2*); **C.***BAX/BCL2*-ratio; **D.** Expression of Caspase 3 (*CASP3*). Data are expressed in n-fold gene expression, except the *BAX/BCL2*-ratio, and are shown with standard deviation bars. *Indicates significant differences with p<0.05; ** = p<0.01. *** =p<0.001.

General Discussion

This study was performed to examine the tolerability of administration of a drug delivery system, and thereby to determine the sustained anti-inflammatory effect of celecoxib when loaded in hydro-gel on the NP cells in an inflamed environment *in vitro*, simulating IVD degeneration.

TNF-α showed a time dependent inflammatory reaction on the nucleus pulposus cells

Results have shown that gene expression of both *Col2* and *Acan* are decreased in pellets collected at day 3. This is in line with our first pilot study, when the measurement was performed on samples collected at day 7. For collagen type 1 there is no increase shown, although in samples collected at day 7 there was a clear increase. This may implicate that the process of inflammation induced by TNF-α is not yet manifest at day 3 to have an effect on gene expression of *Col1*. This is in accordance to the results of a study on the dose responding effect of TNF-α performed on bovine nucleus pulposus cells. After 48 hours, these cells showed

a clear effect on collagen type 2 and aggrecan. However no effect was seen on the expression of Col1 for any used concentration of TNF-α.[24] On the catabolic gene expression, TNF-α showed an upregulation (MMP9, MMP13 and ADAMTS5). Although these effects showed no significance, these results were in line with data of biochemical analysis and histology. These showed a catabolic active environment in pellets treated with TNF-α (data are not published in present document). On the other hand, expression of TIMP1 showed an increase after 3 days, while after 7 days no difference in expression was showed. Expression of both IL-6 and the pro-apoptotic gene CASP3 showed after 3 days an upregulation where on day 7 a downregulation was measured. All together these results suggest that the process of inflammation is a dynamic proses. This is also described in a quantitative analysis of gene expression in a rabbit model of intervertebral disc degeneration by Sobajima et all.[25] Where they performed PCR on nucleus pulposus tissue after inducing inflammation at 3, 6, 12 and 24 weeks after confirmation of degenerative changes by a magnetic resonance imaging (MRI) scan. In this study inflammation was induced by stabbing the discs using a needle. This study showed that gene expression is not following a clear up/down regulation in time, but showed in some genes (double) peak characteristics. This indicates as well that simulating inflammatory environment *in vitro* is complicate and hard to predict. This should be taken into account in future research and it is recommended to perform gene expression measurements on several time-points. In order to define a possible difference between the measured gene expression and the eventual protein production an additional experiment should be performed to measure the produced protein by using ELISA or immunohistochemistry.

No clear anti-inflammatory effect of celecoxib loaded hydro-gel was shown

Concluding on gene expression, celecoxib loaded hydro-gel showed no regenerative effect which was contrary to the expectations. This may have two possible explanations. Loaded hydro-gel secreted inadequate doses of celecoxib. Or celecoxib loaded hydro-gel was non-functional in this *in vitro* experiment. Another study used this hydro-gel in an *in vitro* experiment to determine the release of celecoxib and showed that during the first 10 days there was a period of no release (Figure 19).[14] However, this release experiment was performed in PBS and may be not reliable for the release of the hydro-gel cultured with cells. For future research it may be additional to measure the release of CXB. This could be measured in the culture medium by using an ELISA. This way it can be controlled, if the adverse effects where due to a to low release. For the present study we are able to compare the effects of the loaded hydro-gel with the different concentrations of CXB bolus treatments. This may indicate which concentration is most similar to the concentration celecoxib released from the hydro-gel.

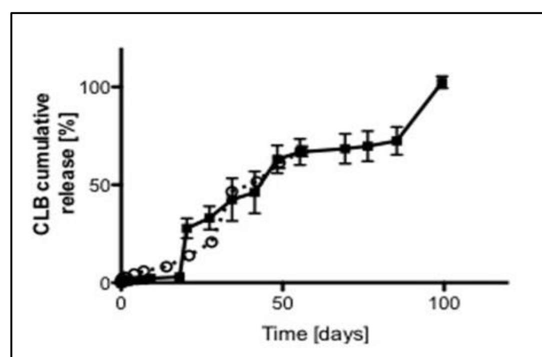

Figure 19 Petit 2014

Unloaded hydrogel showed no negative effects of gene expression of NP cells
In order to investigate the tolerability of the hydrogel administration on nucleus pulposus cells unloaded hydro-gel was tested in the present study. There are studies which indicate that celecoxib loaded hydro-gel was safe, when administrated subcutaneous and intra-articular.[14,26] To the author's knowledge, no data was published of the effects of the unloaded hydrogel *in vivo*. Based on this present study the effects of the unloaded hydro-gel on gene expression showed no contrary results. This suggests that unloaded hydro-gel is safe for in vivo administration. However, to complete this statement an extra condition should be tested were the unloaded hydro-gel is added to pellets only treated with TGF and did not achieve any inflammatory stimulus. This way the effect of the hydrogel can be evaluated on a healthy intervertebral disc.

The effect of the increasing doses of celecoxib bolus treatment
A dose-dependent effect was shown in gene expression of the increasing concentrations of celecoxib. The highest concentration celecoxib (CXB10^-4) showed on *Col2*, *Acan*, *PTGES2* an *Casp3* different gene expression compared to the lower two celecoxib concentrations. This might suggest a negative effect of a too high concentration of celecoxib on the nucleus pulposus cells. A study on the dose response of celecoxib showed that celecoxib loses its anti-inflammatory effect at high doses. Celecoxib was systemically administrated to rats with an induced inflamed paw. Celecoxib showed a significant anti-inflammatory effect when using a dose of 50 mg/kg. This effect was lost when using 100 or 200 mg/kg. These doses provide a plasma concentration of 20-30 microM. The study showed that these effects were due to that celecoxib at high concentration induces an activation of NF-κB and thereby inducing the transcription of NF-κB-dependent genes such as TNF-a.[27] In this present study no negative effects were shown for the lower two concentrations, however a clear regenerative effect has not been seen. This might be due to the effect that the inflammation induced by adding 10 ng/ml TNF-α was too dominating, resulting that celecoxib did not show any effect contrary to the expectations. This is in line with a paper performing quantification of the present TNF-α values in acute and chronic degenerated intervertebral disc. It is not possible to compare these findings directly with the doses of TNF-α used in this experiment. Nevertheless there has been a factor 1000 difference between the amount used in this study and published data of Altun I. et al. [28] For future research it is recommended that the dose of TNF-α should be reduced. Or another possibility is culturing the NPC without TGF-β and TNF-α and in this way a better effect of celecoxib could be seen. Another explanation for the lack of effect of celecoxib is that due to the treatment of the inflammation, the pathway which plays an essential role in regeneration might not be activated. This way using an anti-inflammatory drug, the balance between inflammation and regeneration will be disrupted. A phenomenon described as the "Phoenix Rising" pathway by Li. F. et al. is explained as 'the pathway by which executioner caspases in apoptotic cells promote wound healing and tissue regeneration in multicellular organisms'.[29]

These results and previous performed studies show that a model for evaluate degenerated nucleus pulposus cells is hard to create and replicate. Up to date, there is no good way to

exactly simulate an in vivo situation. Therefore a possible solution is to perform such experiments in vivo.

Highest concentration of ethanol showed an effect of gene expression of NP cells
The results of the gene expression of the pellets treated with the two concentrations of ethanol showed no adverse effects. Contrary to the expectations the highest concentration of ethanol (0,4%) showed an regenerative effect on gene expression of the catabolic genes MMP13 and ADAMTS5. Therefore it was additional in this experiment to take ethanol as a separate control group. Based on the results it is suggestive that no negative effects of celecoxib are caused when diluted with ethanol.

Conclusions

The overall purpose of this project is to translate treatment towards the veterinary patients suffering from discogenic pain by a controlled release of medication via local delivery. The specific aim of this in vitro study was to determine the anti-inflammatory effect of celecoxib, processed in a hydro-gel drug delivery system, on degenerated canine nucleus pulposus cells. It was hypothesiszed that celecoxib ,released from the drug delivery system, has a sustained anti-inflammatory effect. This hypothesis should, based on this present study, be rejected. Further research is needed to determine the effects of celecoxib, both processed in hydro-gel or in bolus treatment. Maybe this should be performed in an in vivo study, because in this present study it is shown that an inflammatory model for the in vitro study of nucleus pulposus cells is inconvenient to reproduce and did not create a reliable environment resembling the in vivo situation in intervertebral disc degeneration.

References

1. Bergknut, N. *et al.* Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. *Vet. J.* **195,** 282–291 (2013).

2. Smolders, L. a. *et al.* Intervertebral disc degeneration in the dog. Part 2: Chondrodystrophic and non-chondrodystrophic breeds. *Vet. J.* **195,** 292–299 (2013).

3. Le Maitre, C. L., Freemont, A. J. & Hoyland, J. A. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res. Ther.* **7,** R732–R745 (2005).

4. Binch, A. L. A. *et al.* Nerves are more abundant than blood vessels in the degenerate human intervertebral disc. *Arthritis Res. Ther.* **17,** 370 (2015).

5. Du, H. *et al.* Dynamic contrast enhanced-magnetic resonance imaging study of the nutrition pathway for lumbar intervertebral disk cartilage of normal goats. *Orthop. Surg.* **3,** 106–12 (2011).

6. Lee, S. *et al.* Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus. *Clin. Biochem.* **42,** 1504–1511 (2009).

7. Walter, B. a *et al.* Inflammatory Kinetics and Efficacy of Anti-inflammatory Treatments on Human Nucleus Pulposus Cells. *Spine (Phila. Pa. 1976).* **40,** 1 (2015).

8. Sainoh, T. *et al.* Interleukin-6 and interleukin-6 receptor expression, localization, and involvement in pain-sensing neuron activation in a mouse intervertebral disc injury model. *J. Orthop. Res.* n/a–n/a (2015). doi:10.1002/jor.22925

9. Funk, C. D. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294,** 1871–5 (2001).

10. Sampey, A. V, Monrad, S. & Crofford, L. J. Microsomal prostaglandin E synthase-1: the inducible synthase for prostaglandin E2. *Arthritis Res. Ther.* **7,** 114–7 (2005).

11. Ruan, D. & So, S.-P. Prostaglandin E2 produced by inducible COX-2 and mPGES-1 promoting cancer cell proliferation in vitro and in vivo. *Life Sci.* **116,** 1–8 (2014).

12. Goldstein, J. L. *et al.* Reduced risk of upper gastrointestinal ulcer complications with celecoxib, a novel COX-2 inhibitor. *Am. J. Gastroenterol.* **95,** 1681–90 (2000).

13. Petit, A. *et al.* Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. *Biomaterials* **53,** 426–436 (2015).

14. Petit, A. *et al.* Release behavior and intra-articular biocompatibility of celecoxib-loaded acetyl-capped PCLA-PEG-PCLA thermogels. *Biomaterials* **35,** 7919–7928 (2014).

15. Séguin, C. A., Pilliar, R. M., Roughley, P. J., & Kandel, R. A. Tumor Necrosis Factor-α Modulates Matrix Production and Catabolism in Nucleus Pulposus Tissue. *Spine* 1940–1948 (2005). Available at: http://ovidsp.tx.ovid.com/sp-3.19.0a/ovidweb.cgi?QS2=434f4e1a73d37e8c740ce3cde7a33785926d9639151ecbef3af7d596823c683413bbf1e78dd12bef6c3d46c0ecf89f91cf92e88e038df4faee6ac56b9e7f91a2cbc5e992c17ce0044c04904bcd18942b455836205be026ab59d0a1b324fd6ffbd61075c32f. (Accessed: 14th April 2016)

16. Teixeira, G. Q. *et al.* A Degenerative/Proinflammatory Intervertebral Disc Organ Culture: An Ex Vivo Model for Anti-inflammatory Drug and Cell Therapy. *Tissue Eng. Part C. Methods* **22,** 8–19 (2016).

17. Emery, P. *et al.* Celecoxib versus diclofenac in long-term management of rheumatoid arthritis: randomised double-blind comparison. *Lancet (London, England)* **354,** 2106–11 (1999).

18. Mwale, F. *et al.* Effect of a Type II Collagen Fragment on the Expression of Genes of the Extracellular Matrix in Cells of the Intervertebral Disc. *Open Orthop. J.* **2,** 1–9 (2008).

19. Yuan, W. *et al.* Establishment of intervertebral disc degeneration model induced by ischemic sub-endplate in rat tail. *Spine J.* **15,** 1050–9 (2015).

20. Wong, P. K. K., Campbell, I. K., Egan, P. J., Ernst, M. & Wicks, I. P. The role of the interleukin-6 family of cytokines in inflammatory arthritis and bone turnover. *Arthritis Rheum.* **48,** 1177–89 (2003).

21. Aderka, D., Le, J. M. & Vilcek, J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* **143,** 3517–23 (1989).

22. Willems, N. *et al.* Biocompatibility and intradiscal application of a thermoreversible celecoxib-loaded poly-N-isopropylacrylamide MgFe-layered double hydroxide hydrogel in a canine model. *Arthritis Res. Ther.* **17,** 214 (2015).

23. Li, W. *et al.* Blocking the function of inflammatory cytokines and mediators by using IL-10 and TGF-β: a potential biological immunotherapy for intervertebral disc degeneration in a beagle model. *Int. J. Mol. Sci.* **15,** 17270–83 (2014).

24. Séguin, C., Pilliar, R., Roughley, P. & Kandel, R. Tumor necrosis factorα modulates matrix production and catabolism in nucleus pulposus tissue. *Spine (Phila. Pa. 1976).* (2005).

25. Sobajima, S., Shimer, A. & Chadderdon, R. Quantitative analysis of gene expression in a rabbit model of intervertebral disc degeneration by real-time polymerase chain reaction. *Spine J.* (2005).

26. Petit, A. *et al.* Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. *Biomaterials* **53,** 426–36 (2015).

27. Niederberger, E. Celecoxib loses its anti-inflammatory efficacy at high doses through activation of NF-k B. *FASEB J.* **15,** 1622–1624 (2001).

28. Altun, I. Cytokine profile in degenerated painful intervertebral disc: variability with respect to duration of symptoms and type of disease. *Spine J.* (2016). doi:10.1016/j.spinee.2016.03.019

29. Li, F. *et al.* Apoptotic cells activate the 'phoenix rising' pathway to promote wound healing and tissue regeneration. *Sci. Signal.* **3,** ra13 (2010).

30. Shim, W. S., Kim, S. W. & Lee, D. S. Sulfonamide-based pH- and temperature-sensitive biodegradable block copolymer hydrogels. *Biomacromolecules* **7,** 1935–41 (2006).