Utrecht University

The role of the PTHrP-IHH feedback loop in canine intervertebral disc degeneration

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Abstract

Introduction - The vertebral column consists of vertebrae interconnected by intervertebral discs (IVDs). These IVDs consist of a nucleus pulposus (NP) surrounded by an annulus fibrosus (AF), and provide the flexibility and stability of the spine. IVD degeneration can cause IVD disease and is a relatively common reason for canine euthanasia. Current treatments for IVD degeneration and IVD disease aim to reduce the pain rather than repairing the IVD. Therefore, the development of regenerative treatments which do restore the biomechanical function is very important. To develop such a regenerative treatment, further knowledge of the pathogenesis of IVD degeneration and the pathways involved is required. The aim of this study was to determine the role of the Parathyroid Hormone-Related Peptide (PTHrP)-Indian Hedgehog (IHH) feedback loop in canine IVD degeneration.

Methods - Canine IVDs with different Thompson scores were immunohistochemically stained for IHH. The mean percentage positive cells per Thompson grade was assessed in the NP and the correlation between the degenerative grades and IHH protein expression in the NP was determined. Also quantitative PCR for *IHH, PTHrP* and their receptors Smoothened (*SMO), Patched (PTCH)* and Parathyroid Hormone-receptor 1 (*PTHR1)* was performed on notochordal cells (NCs) that were cultured in monolayers and in clusters (culture day 0, 2, 4, 6, 8, 10, n = 8). To compare the *in vitro* qPCR results with the *in vivo* situation, cDNA from (non-digested, non-cultured) NC-rich NP tissue of six different NCD dogs was used as a control.

Results - The mean percentage IHH positive cells decreased from Thompson grade I to II, where it was the lowest. From Thompson grade II to V this percentage increased. There was a significant positive correlation between IHH protein expression and IVD degeneration grade. In addition, NC gene expression of *IHH, PTHrP* and its receptor *PTHR1* was decreased in the cultured NCs compared with the control group, while gene expression of the IHH receptors *SMO* and *PTCH* was increased compared with the control group.

Conclusion - This study demonstrated a significant positive correlation between IHH protein expression and IVD degeneration grade, which may indicate that IHH stimulates IVD degeneration. Future studies are, however, needed to further elucidate the exact role of the PTHrP-IHH feedback loop in IVD degeneration and to determine if altering this pathway could be a point of engagement for the development of a regenerative treatment.

Keywords: Intervertebral Disc; Intervertebral disc degeneration; Nucleus pulposus; Indian Hedgehog; Parathyroid Hormone-Related Peptide; Canine;

Introduction

The vertebral column consists of vertebrae interconnected by intervertebral discs (IVDs). The IVD transmits compressive forces between the vertebral bodies, permits movement between vertebrae and provides flexibility and stability to the spine.[1,2] In its healthy and fully hydrated state, the IVD permits a few degrees of extension, flexion and lateral bending. All these small movements from all IVDs summed together allow movement of the spine.[3]

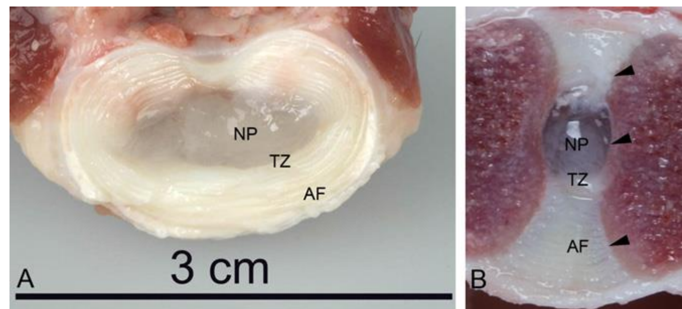

Figure 1 Transverse (A) and sagittal (B) sections through a L5–L6 intervertebral disc of a mature non-chondrodystrophic dog, showing the nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF), and end plates (arrowheads).[2]

A healthy IVD consists of four distinct parts, namely, the nucleus pulposus (NP), the annulus fibrosus (AF), the transition zone (TZ) and the vertebral end plates (EPs) (Figure 1). The NP lies eccentrically in the IVD, is bean-shaped, translucent and semigelatinous. The healthy NP is composed of two types of fibres, there is a complex network of proteoglycans interwoven in randomly organized collagen type II fibres. The proteoglycans are negatively charged and hydrophilic and therefore draw water into the NP through osmosis. As a result, the matrix of the NP is highly hydrated (80% water) and a high intradiscal pressure is created which allows the IVD to function as a biomechanical cushion.[4] In a healthy IVD, the main cell type in the NP are the notochordal cells (NCs). NCs are large cells found in clusters and can be recognized by their cytoplasmatic vesicles.

The AF surrounds the NP and is composed of an organized network of concentric fibrous lamellae. Each lamellae consists of concentric rings of collagen fibrils combined with elastic fibres and covered with proteoglycans. In the outer layers of the AF, collagen type I is predominant, whereas the inner layers contain mainly collagen type II. The matrix of the AF is hydrated and contains 60% water.[4] The outer layers of the AF contain fibroblast-like cells, while the inner layers contain a mix of chondrocyte-like cells and fibroblasts. This more mucoid or cartilaginous inner layer of the AF is also known as the TZ and interconnects the NP and the AF. The fibres of this TZ make a strong connection with cartilaginous EPs, which form the cranial and caudal borders of the IVD.[2] In dogs, the EP is composed of chondrocyte-like cells (CLCs) surrounded by layers of matrix. Biochemically the composition of the EP resembles that of articular cartilage. The matrix is mainly composed of water (50-80%), proteoglycans and collagen type II.[2]

IVD degeneration

Degeneration of the IVD can cause IVD disease, which is a relatively common reason for euthanasia in dogs. However, incidental IVD degeneration is also found in dogs without clinical signs.[5,6] IVD degeneration is characterized by biochemical changes in the composition and the loss of mechanical integrity.[1] It is a multifactorial and complex process and in dogs, the exact pathophysiology is yet to be explored. However, it is known that IVD degeneration in dogs is similar to the degeneration process in human IVDs.[2] IVD degeneration has been associated with chronic biomechanical stress, trauma, altered

levels of enzyme activity, inadequate metabolite and nutrient transport, cell senescence and cell death, changes in matrix, changes in water content and genetic predisposition.[2]

With IVD degeneration, a phenotypical transition of NP cells takes place: the NCs are gradually replaced by the smaller CLCs. The CLCs produce a different extracellular matrix than the NCs, this matrix resembles hyaline cartilage and comprises mostly disorganised collagen type I fibres. While the collagen I content increases and the GAG and collagen II content decreases, the matrix becomes more rigid and the water content decreases. These changes in the matrix can also result in calcification of the NP and make that the IVD loses its ability to fully fulfil its biomechanical function.[2,7] As a result of the changes in the matrix, tears and clefts can occur in the NP.
While the IVD is avascular, the nutrient supply to the NP takes place through diffusion. Therefore, another effect of these changes in the NP matrix is that the diffusion of nutrients is impaired. This impaired nutrient diffusion leads to cellular changes and cell senescence or even apoptosis, which again results in matrix remodelling. During degeneration of the AF, there is an ingrowth of CLCs from the TZ, the concentric lamellae disorganise which makes the AF weaker, and more cross-links between the collagen fibres are formed which prevent lamellar movement in the AF.[2,8]
All these changes together form an vicious circle of structural and functional degeneration and inadequate repair which eventually may result in structural changes of the IVD and even the vertebral bodies, including disc prolapse or herniation, annular tears and endplate disruption (Figure 2).[2]

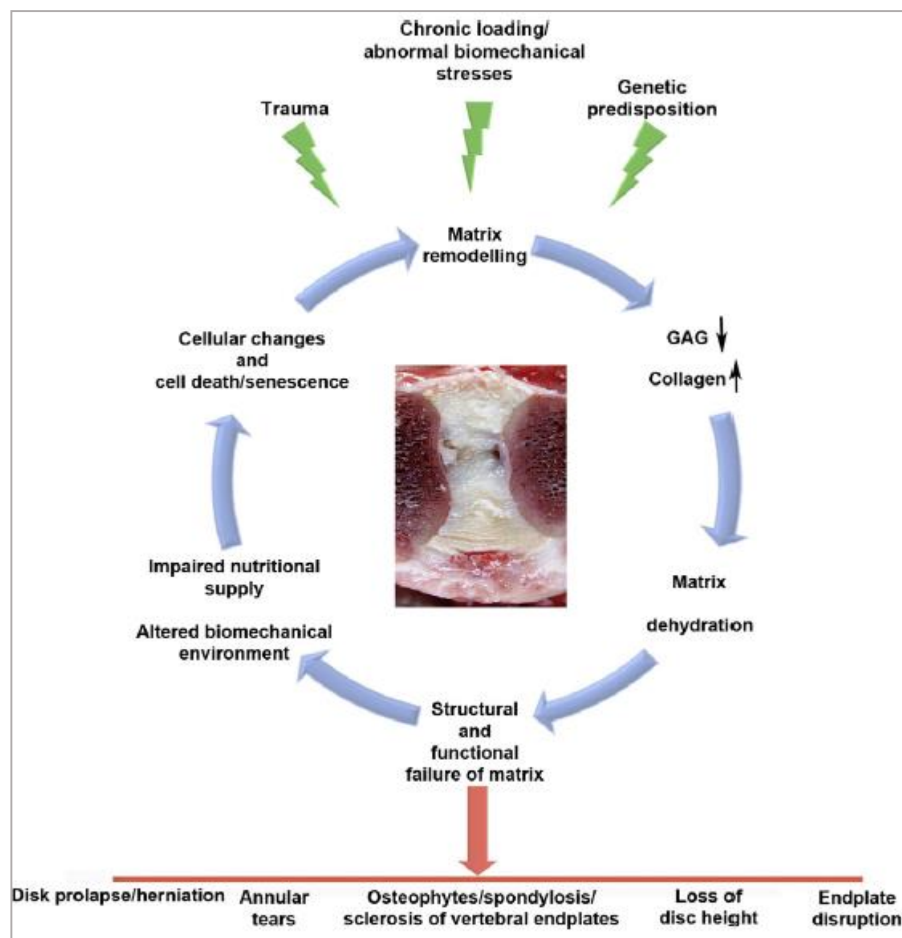

Figure 2 Schematic representation of the pathophysiology of intervertebral disc degeneration.[2]

For objective and reproducible macroscopic grading of IVD degeneration, the Thompson score can be used (Table 1). This score divides IVD degeneration into five grades based on structural and cellular changes in the IVD. Grade I stands for a healthy IVD and grade V represents end stage degeneration (Figure 3).[9]

Grade	Nucleus pulposus	Annulus fibrosus	End plates	Vertebral bodies
I	Bulging gel	Discrete fibrous lamellae	Hyaline, uniform thickness	Rounded margins
II	White fibrous tissue peripherally	Mucinous material between lamellae	Irregular thickness	Pointed margins
III	Consolidated fibrous tissue	Extensive mucinous infiltration: loss of annular-nuclear demarcation	Focal defects in cartilage	Early chondrophytes or osteophytes at margins
IV	Horizontal clefts parallel to end plate	Focal disruptions	Fibrocartilage extending from subchondral bone; irregularity and focal sclerosis in subchondral bone	Osteophytes < 2mm
V	Clefts extended through nucleus and annulus		Diffuse sclerosis	Osteophytes > 2mm

Table 1 Description of the five grades of the Thompson grading scheme used for grading of IVD degeneration. Grade I represents a healthy IVD, grade V is end stage degeneration.[9]

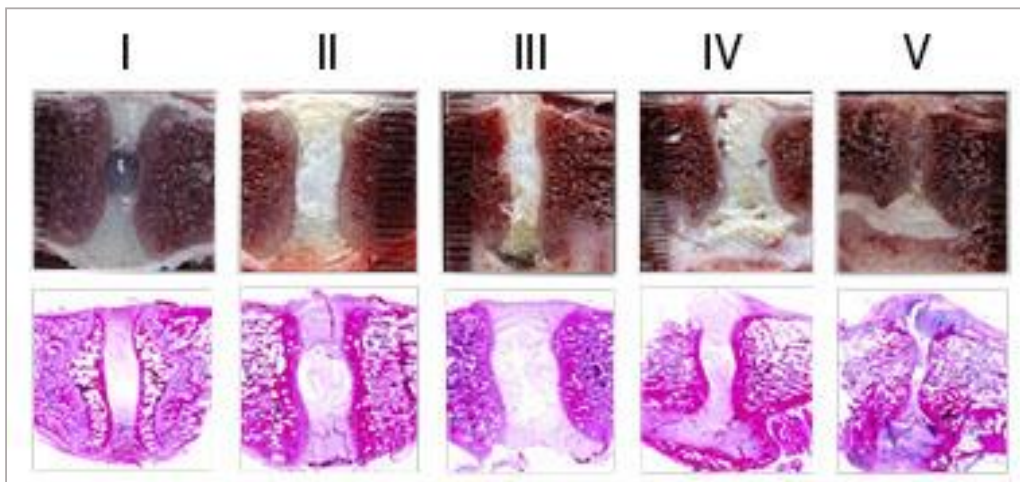

Figure 3 macroscopic (top) and histo-pathological (bottom) images showing the different Thompson grades. Far left the healthy grade I and from left to right the severity increases. Modified from N. Bergknut *et al*, 2013.[2]

IVD degeneration can occur in all dog breeds, but is more common in chondrodystrophic (CD) than in non-chondrodystrophic (NCD) breeds. In CD dogs, the endochondral ossification, primarily in the long bones, is disturbed which results in disproportionally short limbs.[10] Examples of CD breeds are the Beagle, miniature Schnauzer, Dachshund and the Cavalier King Charles Spaniel. The age at which the first changes in the IVD can be seen also differs between CD and NCD breeds. In CD breeds, the replacement of NCs start at the age of three months and is usually completed before one year of age. In NCD breeds on the other hand, this replacement occurs later in life, typically after five years of age. Moreover, the NCs remain the abundant cell type throughout the entire life of NCD breeds.[10]

Current treatments for IVD degeneration and IVD disease aim at reducing pain rather than repairing the IVD.[1,11] While none of these treatments restore the biomechanical function of the IVD, the development of regenerative treatments which do restore the biomechanical function is very important. To develop such a regenerative therapy for IVD degeneration, further knowledge of the pathogenesis of IVD degeneration and the pathways involved is required.[12]
The changes in cell types during IVD degeneration can be related to changes in numerous signalling pathways, including Wnt/β-catenin signalling, plasmin signalling, extracellular matrix remodelling and plasminogen activator-urokinase-signaling.[13] However, there are other pathways in IVD degeneration that have not been studied yet, including the parathyroid hormone-related peptide (PTHrP) and Indian Hedgehog (IHH) pathway.

PTHrP-IHH pathway
There is not much known about the role of the PTHrP-IHH feedback loop in the IVD. The effect of the PTHrP-IHH feedback loop in the growth plate, however, has been extensively studied. It is known that this loop provides important growth-restraining feedback in longitudinal bone growth. It plays an important role during pre- and postnatal endochondral bone formation by determining the pace and synchrony of chondrocyte transition from proliferation into hypertrophic differentiation.[14]

PTHrP is a protein produced in a large variety of tissues, including heart, lungs, placenta and cartilage. It has been demonstrated that PTHrP has a wide auto-/ paracrine function.[15] PTHrP binds and activates the PTH/PTHrP receptor PTHR1, which is also activated by parathyroid hormone (PTH). PTH is an important regulator of bone remodelling and of the calcium/phosphate metabolism. Therefore, PTHR1 plays a role in both the auto-/paracrine functions of PTHrP and the endocrine functions of PTH.[16] In the growth plate, PTHrP is secreted by periarticular chondrocytes and inhibits chondrocyte differentiation and maturation.[17] While PTHrP inhibits chondrocyte maturation, IHH promotes chondrocyte differentiation and proliferation. IHH is a protein produced by pre-hypertrophic chondrocytes and also stimulates the production of PTHrP. Because PTHrP inhibits chondrocyte differentiation, it keeps the chondrocytes in a proliferative state thus delaying IHH production. Therefore, PTHrP and IHH are part of a negative feedback loop in which PTHrP inhibits IHH and IHH stimulated PTHrP.[18]

IHH is a potent inducer of proliferation and hypertrophy of chondrocytes.[14] The receptor of IHH, Patched (Ptch), inhibits transmembrane transducer Smoothened (Smo). After binding of IHH, Ptch loses this inhibition and Smo becomes activated. Through a complex cascade, Smo recruits and activates Gli1 proteins, which translocate to the nucleus and act as transcription factors for IHH target genes, for example PTHrP.[19] Activation of this IHH signalling also leads to the expression of Ptch, creating a negative feedback loop that regulates the duration and the level of IHH signalling. In the absence of IHH, Ptch inhibits the function of Smo and induces the formation of various phosphorylating kinases. These kinases phosphorylate Gli1, which results in proteasomal degradation of Gli1 and cleavage of Gli2 and Gli3. GLi2 and GLi3 also translocate to the nucleus, but instead of activating, they downregulate IHH target gene expression (Figure 4).[20,21]

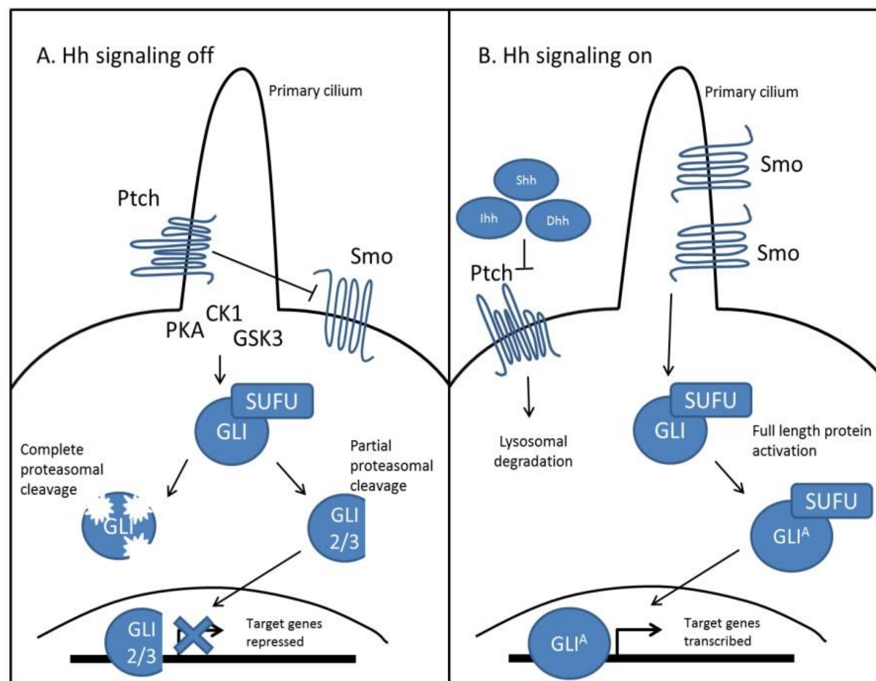

Figure 4 Indian Hedgehog (IHH) signaling pathway. **(A)** Without IHH binding to Patched (Ptch) **(B)** With binding of IHH to Ptch. [19]

Tryfonidou *et al.* (2010) found that there was a difference in the amount and ratio of PTHrP and IHH between large-breeds and small-breed dogs in the growth plate. In large breeds, more IHH is present while PTHrP levels are low. However, in small breeds the PTHrP-IHH ratio is reversed, there is proportionately more PTHrP than IHH. It is likely that these differences between large-breeds and small-breeds in the PTHrP-IHH pathway contribute to differences in growth rate and growth plate morphology. Growth plates of large-breeds are thicker and have larger proliferative and hypertrophic zones. Tryfonidou *et al*. (2010) hypothesized that the PTHrP-IHH feedback loop in large breeds is changed whereby IHH is upregulated and PTHrP is relatively downregulated. As a result, the effects of IHH on the regulation of chondrocyte proliferation and hypertrophy becomes predominant.[14]

It has also been demonstrated that IHH plays an important role in the end plate of the IVD. Wang *et al.* (2015) propose that IHH contributes to endplate degeneration, since the expression of cartilage degeneration markers increased with an IHH gain-of-function *in vitro* experiment.[22] Another study suggests that IHH forms a key signal in the developing human vertebral endplate, where it regulates the growth and differentiation of cells.[23]
The IHH pathway promotes chondrocyte hypertrophy in cartilage, which leads to calcification.[24] Previous studies have also shown that activation of IHH plays an important role in osteoarthritis (OA).[17,24–28]

Thus, there is much known about the PTHrP-IHH pathway and the effect it has in other tissues, however, little is known about what this pathway does in the IVD. In this study the role of the PTHrP-IHH feedback loop in IVD degeneration was studied.

Aim of this study

The aim of this study was to determine the role of the PTHrP - IHH feedback loop in canine IVD degeneration. For this purpose, canine IVDs with different Thompson scores were immuno-histochemically stained for IHH. The mean percentage IHH positive cells per Thompson grade were assessed in the NP and the correlation between the Thompson grade and IHH protein expression in the NP was determined. Also quantitative PCR for *IHH, PTHrP* and their receptors *SMO, PTCH* and *PTHR1* was performed on notochordal cells (NCs) that were cultured in monolayers and in clusters (culture day 0, 2, 4, 6, 8, 10). It was hypothesized that IHH expression will increase during IVD degeneration, since IVD degeneration resembles osteoarthritis and previous work showed an increase in IHH expression during osteoarthritis.[17,24–28] In addition, it is known that IHH stimulates chondrocyte hypertrophy, which leads to calcification, a process that is also common in IVD degeneration.[2,7]

Materials and methods

Immunohistochemistry

Thirty-seven thoracolumbar or lumbosacral IVD samples from fifteen randomly selected canine cadavers were readily available from a previous study by Bergknut *et al.* (2013), where they were used to develop a histological grading scheme for the classification of canine IVD degeneration.[12] The dogs varied in gender (5 male, 10 female), age (16-192 months) and breed (5 CD, 10 NCD). All dogs were client-owned dogs offered for pathologic research to the Pathobiology Department (Faculty of Veterinary Medicine, Utrecht University) or had already been euthanized for unrelated research studies approved by the Utrecht University Ethics Committee. The samples were divided into five different groups based on their Thompson degeneration score (Table 2). These scores were based on the morphology of midsagittal sections of the IVD: score I is healthy and score V represents end stage degeneration.[2] From the samples, sections had already been made, suitable for immunohistochemistry.

Thompson score	I	II	III	IV	V
Number of samples	8	7	8	7	7

Table 2 Overview of the number of samples per Thompson score used for immunohistochemistry.

For the immunohistochemical staining of IHH an ImmunoCruz™ goat LSAB Staining kit sc-2053 (Santa Cruz Biotechnology, Santa Cruz, USA) was used according to the manufacturer's instructions.
First, the sections were dewaxed using two rounds of xylene and rehydrated using graded alcohol (96%, 80%, 70% and 60%). These steps were performed for 5 minutes each. After the dewaxing and rehydration, the sections were washed in PBS with 0.1% Tween (PBST) for 5 minutes. Then the sections were placed in a 10mM citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) and placed in a preheated water bath. The temperature of the water bath was raised from 37°C till 70°C and the sections were incubated for 30 minutes. After 30 minutes, the sections were removed from the water bath and were left to cool down for 20 minutes, still remaining in the citrate buffer. After cooling down, circles were made around the IVD tissue using an ImmEdge™ pen (Vector Laboratories, Burlingame, USA) to ensure that the liquids, which would be added later in this process, would remain on the tissue.

After washing the sections for 2x5 minutes in PBST, excessive liquid was aspirated using a paper tissue. To block endogenous peroxidase activity, two drops of peroxidase block were applied to the sections for 5 minutes. Subsequently, the sections were washed using PBST for 2x5 minutes and excessive liquid was removed. Following this washing, two drops of serum block were placed on the sections, which were left to incubate for 30 minutes. During these 30 minutes, the primary antibody for IHH (sc-1196) was diluted in serum block to create a 1:100 dilution. After the 30 minutes, the serum block was removed by tapping the sections on a tissue on the table and per section 100µl of this antibody-serum block mix was applied. The sections were then incubated overnight at 4°C.

The following day, the sections were washed with PBST for 2x5 minutes and excessive liquid was aspirated. Two drops of secondary antibody were placed onto the sections. After incubating the sections for 30 minutes, they were washed with PBS for 2x5 minutes and excessive liquid was removed. Two drops of HRP-Streaptavidin Complex were added afterwards and the sections were incubated for 30 minutes. After two five minute washes with PBS, 100µl liquid DAB chromogen, diluted in DAB substrate buffer (Dako North America, Carpinteria, USA), was added per section and the sections were incubated for 5 minutes. Next, the sections were rinsed with tap water for 15 minutes. Haematoxylin was applied for 10 seconds, where after the sections were again washed with tap water for 15 minutes. To dehydrate the sections, graded alcohol (60%, 70%, 80%, 96%, 96% and 100%) and two rounds of xylene were used. Each step was performed for 5 minutes. Finally, the sections were mounted using VectaMount™ Permanent Mounting Medium H-5000 (Vector Laboratories, Burlingame, USA).

To digitalize the stained sections, photographs were taken using a Leica DFC420C digital camera (Leica Microsystems, Wetzlar, Germany) mounted to a BX60 microscope (Olympus Corporation, Tokyo, Japan) and the Leica Application Suite (V4.2) software package at a 20x magnification. For each section, the end plate was used as positive control. Normal mouse IgG was used as negative control. Per section, four regions of the nucleus pulposus were randomly selected for quantification. The percentage of IHH positive cells over the total number of cells was counted manually (blinded) using Photoshop CC 2014 by one observer.

qPCR

cDNA from cervical (C2-T1) and lumbar (L1-S1) NC-rich NPs from eight NCD dogs was readily available from a previous study by Smolders *et al.* (2012).[29] The dogs varied in age (16-18 months). All animals had already been euthanized for unrelated research studies approved by the Utrecht University Ethics Committee. Ten NPs were collected from each dog, all the NPs from one dog were pooled, the NP tissue was digested and NCs were collected.[29] The obtained NCs were cultured from each dog in both their original cluster-like conformation and as single cells in monolayer. These cells were collected at days 0, 2, 4, 6, 8 and 10 and for each of time point cDNA was generated. To compare the *in vitro* qPCR results with the *in vivo* situation, cDNA from (non-digested) NP tissue from six different CD and NCD dogs was used. This cDNA was also readily available from the same study by Smolders *et al.* (2012).[29] These dogs varied in age (13-60 months) and breed (6 CD, 6 NCD). From each dog cervical (C2-T1) and thoracolumbar (T10-S1) naïve NP tissue was available, which was snap frozen and stored at -70 °C.

To normalize the gene expression of the target genes (Indian Hedgehog (*IHH*), Patched (*PTCH*), Smoothened (*SMO*), parathyroid hormone-related peptide (*PTHrP*) and parathyroid hormone-related peptide receptor (*PTHR-1*)) four stably expressed reference genes (Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Ribosomal protein S19 (*RPS19*) and succinate dehydrogenase complex subunit A (*SDHA*)) were used. The primer sequences are shown in Table 3.

Gene	Primer sequence	Product Size (bp)	PCR temperature condition (°C)
IHH	Fwd: 5'- TCACCACTCAGAGGAGTCG -3' Rev: 5'- GTGCTCAGACTTGACGGAG -3'	172	60
PTCH	Fwd: 5'- CCTCCTCATATTTGGGGC -3' Rev: 5'- CACCTTCTTCTTTCGGGG -3'	158	57
SMO	Fwd: 5'- CTATGTGCTGTGCCAG -3' Rev: 5'- ATCACTCTGCCCAGTC -3'	214	62
PTHrP	Fwd: 5'- GTGTTCCTGCTGAGCTACTCG -3' Rev: 5'- ATGGGTGGTCGCCTTCTA -3'	451	66.5
PTHR-1	Fwd: 5'-GACCACATCCTTTGCTGG-3' Rev: 5'-CAAACACCTCCCGTTCAC-3'	217	51
HPRT	Fwd: 5'-AGCTTGCTGGTGAAAAGGAC-3' Rev: 5'-TTATAGTCAAGGGCATATCC-3'	104	56 + 58
GAPDH	Fwd: 5'-TGTCCCCACCCCCAATGTATC-3' Rev: 5'-CTCCGATGCCTGCTTCACTACCTT-3'	100	58
RPS19	Fwd: 5'-CCTTCCTCAAAAAGTCTGGG-3' Rev: 5'-GTTCTCATCGTAGGGAGCAAG-3'	95	61 + 63
SDHA	Fwd: 5'-GCCTTGGATCTCTTGATGGA-3' Rev: 5'-TTCTTGGCTCTTATGCGATG-3'	92	51

Table 3 Primer sequences used for quantitative PCR

The undiluted cDNA was 5x diluted and a standard line was made. This standard line consisted of a pool of cDNA from all the samples. S1 contained the highest standard and a serial dilution of 4x was made toward S7 by pipetting 40 µL liquid from the previous well into the next well and adding 120 µL MQ. The remaining 5x diluted cDNA was diluted another 5x to yield 25x diluted cDNA. Master mix was made with 6 µL forward primer, 6 µL reverse primer, 129 µL MQ and 702 µL iQTTM SYBR green Supermix (Bio-Rad, Veenendaal, The Netherlands).

According to the plate setup, 6 µL master mix and 4 µL of the 25x diluted cDNA was pipetted in a 384 wells plate after which the plate was centrifuged in an Eppendorf Centrifuge 5804 (Eppendorf AG, Hamburg, Germany). Finally, the plate was put into the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) at the right temperature for a 2- step protocol.

For determination of the relative quantitative gene expression, the $E^{\Delta\Delta Ct}$ method was used.

ΔCt-values were calculated for each target gene by subtracting the mean Ct-value of the reference genes from the Ct-value of the target genes ($\Delta Ct = Ct_{mean\ ref} - Ct_{target}$). Ct-values of the control group (NCD NP tissue) were used as calibrator. The $E^{\Delta\Delta Ct}$-value was calculated and for each target gene, the mean n-fold changes and standard deviations in gene expression were calculated.

Statistical analysis

For the statistical analysis, SPSS Statistics 22 was used. First it was examined if the data was normally distributed using the Shapiro-Wilk test (n<2000). On the normal distributed data, a one-way ANOVA was performed. For the non-normal distributed data, the Kruskall Wallis was used. When significances of P<0.05 were found the Mann-Withney U test was used. For both the normal and the non-normally distributed data, the Benjamini Hochberg correction was used as post hoc test to correct for multiple comparisons.

Partial correlations were calculated to determine the relationship between the degenerative grades and the percentage IHH positive cells per grade, while correcting for the donors used in the study. In all tests p-values < 0.05 were considered significant.

Results

Immunohistochemistry

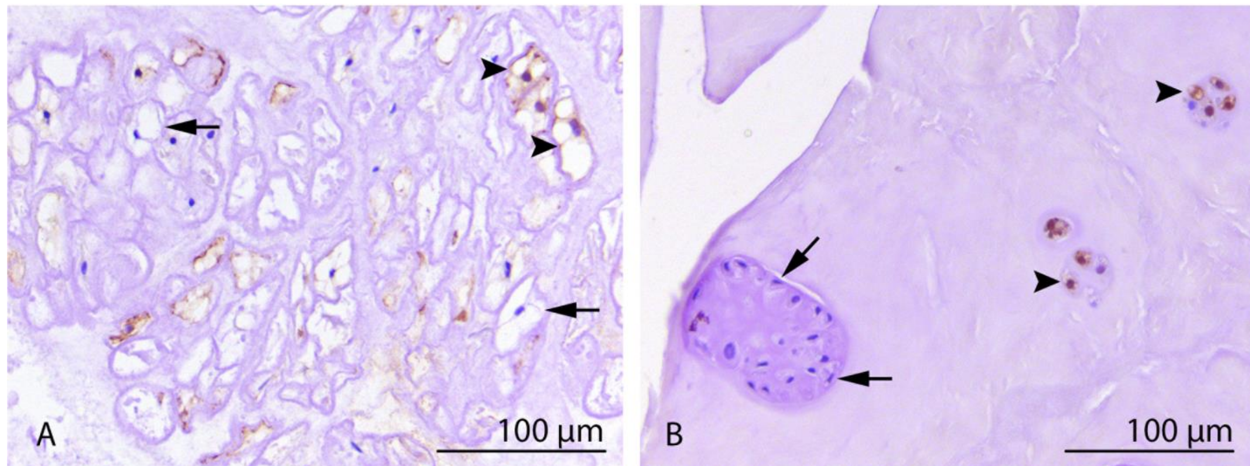

Figure 5 Results of the immunohistochemical staining for Indian Hedgehog (IHH). **(A)** Canine nucleus pulposus (NP) tissue from an intervertebral disc (IVD) with Thompson grade I. Typical large vacuolated notochordal cells (NCs) are visible. **(B)** Canine NP tissue from an IVD with Thompson grade V. Typical smaller, non-vacuolated and clustered chondrocyte-like cells (CLCs) are visible. Arrow heads indicate cells positively stained for IHH, arrows indicate cells negative for IHH.

Thirty-seven thoracolumbar or lumbosacral IVD samples from fifteen randomly selected canine cadavers were used for analysis. The samples were divided into five different groups based on their Thompson degeneration scores. The grade I samples had an average IHH expression in the NP of 28.98%, grade II of 4.25%, grade III of 10.71%, grade IV of 13.13% and grade V of 39.53% (Figure 6).

The IHH protein expression was significantly higher in the NP of Thompson score grade I samples when compared to grade II samples ($P<0.05$). The IHH protein expression was also significantly higher in the NP of Thompson score grade V when compared to grade II, III and IV samples ($P<0.05$).

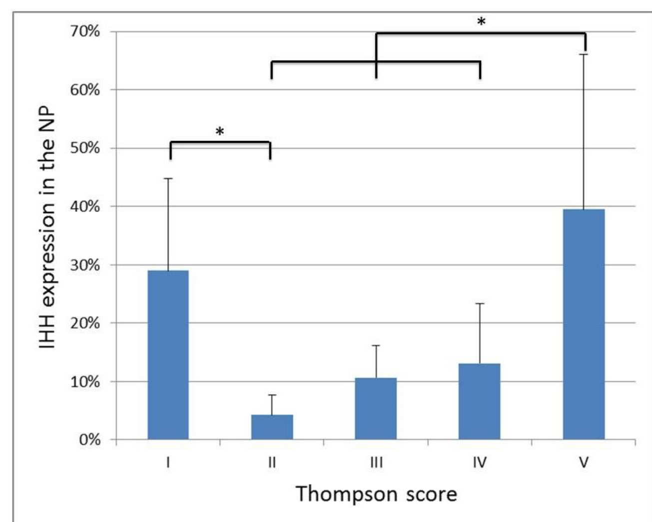

Figure 6 Indian Hedgehog (IHH) protein expression in the canine nucleus pulposus (NP) as mean percentage positive cells per Thompson grade. *: $P<0.05$, $n=37$

There was no significant correlation found between the degenerative grades (Thompson score I-V) and the IHH protein expression in the NP (Figure 7). However, when all grade I samples, which comprised the healthy NC-rich NPs, were removed from the analysis, a strong and significant positive correlation was found between the amount of IHH and the degenerative grade (Thompson score II-V, $R= 0.570$, $P<0.01$), indicating that in CLCs, there is a strong and significant positive correlation between IHH expression and IVD degeneration.

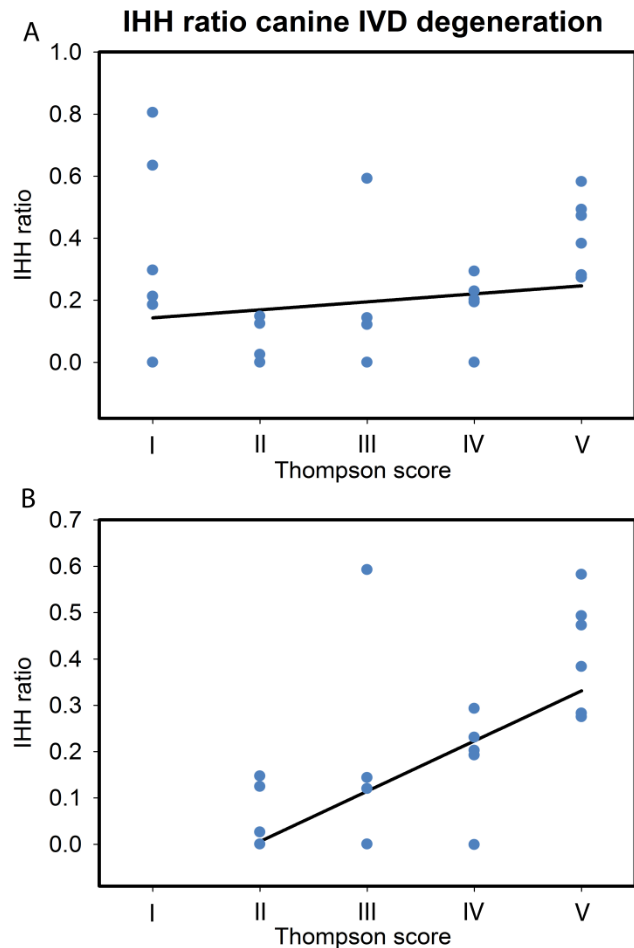

Figure 7 Correlation between the Indian Hedgehog (IHH) ratio and the degenerative grade of the IVD, displayed in Thompson scores. **(A)** No correlation exists when all grades were taken into account. **(B)** A significant correlation ($R= 0.570$, $P<0.01$) was found between IHH expression and Thompson grade II-V. $n=37$

qPCR

To interpret the qPCR output, cells from naive (non-digested, non-cultured) NP tissue of six different NCD dogs was used as a control (NCD NP). The qPCR showed a decrease in *IHH* gene expression for both the cultured single NCs and the NC clusters for every point in time, when compared with the NCD NP control group. (Figure 8A). The control group showed significantly higher *IHH* gene expression than the NC clusters at day 0, 4, 6, 8 and 10 (P<0.01).

Expression of the IHH receptor, *PTCH*, was significantly increased in the NC clusters at day 0 and the single NCs at day 2 when compared to the control group (Figure 8B). In the single NCs, *PTCH* gene expression increased from day 0 until day 2, but thereafter decreased. *PTCH* expression was significantly lower in control group than in the NC clusters at day 0 and the single NCs at day 2 (P<0.01). The CD NP cells had a significantly higher *PTCH* gene expression than the NCD control group (P<0.01).

Compared to the control group, expression of *SMO* was significantly increased in the NC clusters at day 10 (P<0.05) (Figure 8C). The NC clusters of day 10 also showed a significant higher *SMO* gene expression than the NC clusters at day 0, 2 and 4 (P<0.05).

PTHrP expression of the NC clusters at day 0 was more than ten times higher than the gene expression of the control group (P<0.01) (Figure 8D). However, the gene expression decreased rapidly and the *PTHrP* gene expression of day 10 was significantly lower than *PTHrP* gene expression of day 0 (P<0.01). Moreover, the NC clusters of day 0 were also significantly higher in *PTHrP* gene expression than the NC clusters of day 4, 6 and 8 (P<0.01).

At day 0, the gene expression of the PTHrP receptor, *PTHR1*, was higher than the control for both the NC clusters and the single cells (P<0.05) (Figure 8E). The *PTHR1* gene expression was significantly higher in the control group than in the NC clusters at day 0, 2, 4, 6, 8 and single NCs at day 2, 4, 6, 8 and 10 (P<0.05). Both the single NCs and the NC clusters had a significantly higher *PTHR1* gene expression than all other groups (P<0.05). The *PTHR1* gene expression in the NC clusters of day 2 only significantly differed with the NC clusters of day 10 (P<0.05).

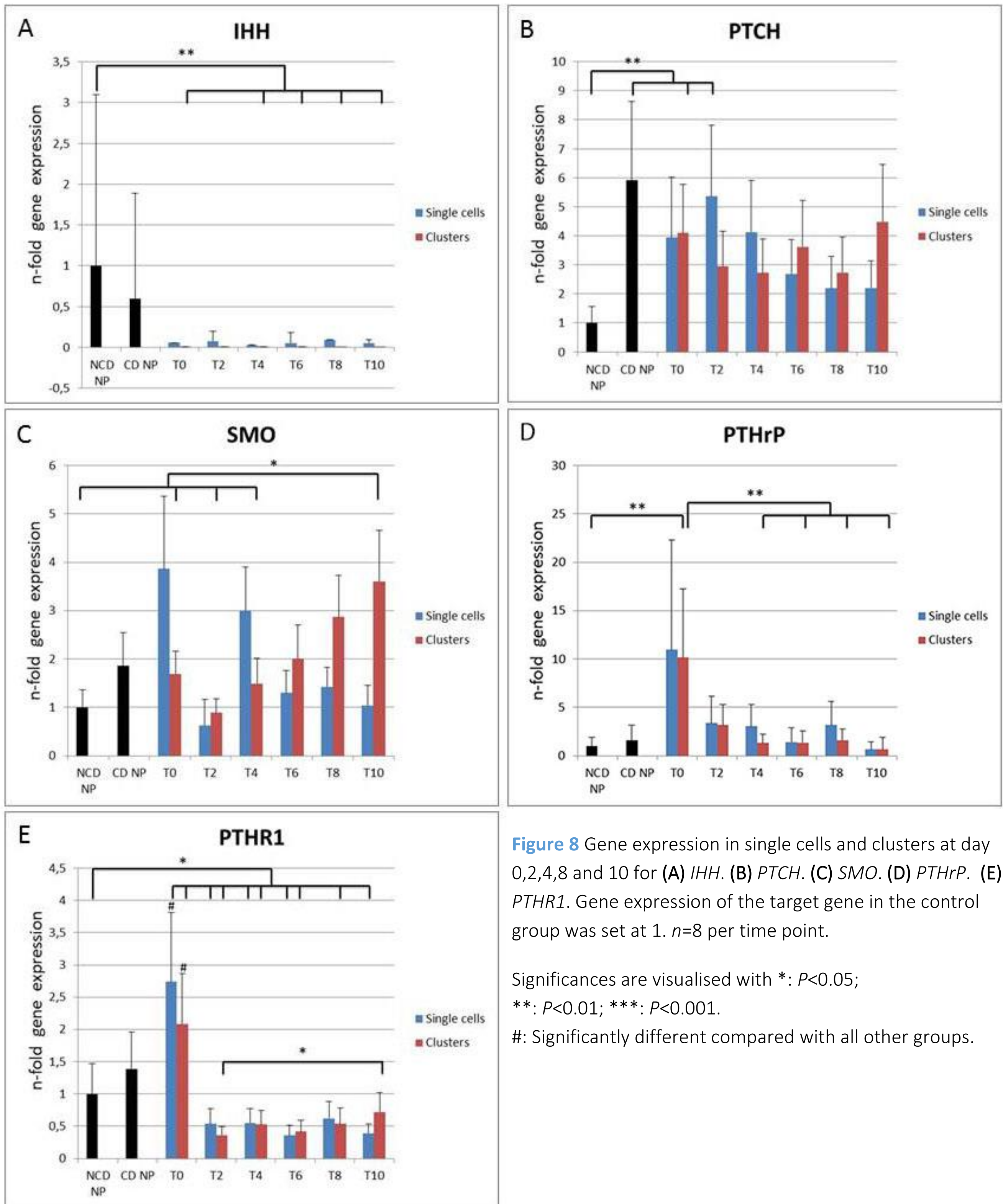

Figure 8 Gene expression in single cells and clusters at day 0,2,4,8 and 10 for **(A)** *IHH*. **(B)** *PTCH*. **(C)** *SMO*. **(D)** *PTHrP*. **(E)** *PTHR1*. Gene expression of the target gene in the control group was set at 1. *n*=8 per time point.

Significances are visualised with *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.
#: Significantly different compared with all other groups.

Discussion

IVD degeneration can cause IVD disease and is a relatively common reason for canine euthanasia.[5,6] While current treatments for IVD degeneration and IVD disease aim to reduce the pain rather than repairing the IVD, the development of regenerative treatments which do restore the biomechanical function is very important.[1] To be able to develop regenerative treatments, further knowledge of the pathogenesis of IVD degeneration is required.[12] The changes in cell type during degeneration can be related to changes in numerous signalling pathways, including the PTHrP-IHH feedback loop. This feedback loop is extensively described in the growth plate but its role in the IVD is yet to be explored. The aim of this study was to determine the role of the PTHrP-IHH feedback loop in canine IVD degeneration. Therefore, canine IVDs with different Thompson scores were immunohistochemically stained for IHH. The mean percentage IHH positive cells per Thompson grade were assessed and the correlation between the Thompson grade and IHH protein expression in the NP was determined. Also quantitative PCR for *IHH, PTHrP* and their receptors *SMO, PTCH* and *PTHR1* was performed on NCs that were cultured in monolayers and in clusters (culture day 0, 2, 4, 6, 8, 10). The latter represents the transition from healthy NCs towards a fibroblast-like phenotype (i.e. early degeneration). It is well-known that cells dedifferentiate during *in vitro* culture, which is also characterized by an altered gene expression.[30]

This study showed significantly lower IHH levels in NPs from healthy (Thompson grade I) IVDs than in NPs from early degenerated (Thompson grade II) IVDs and a higher IHH protein expression in NPs from Thompson grade V IVDs than in NPs from Thompson grades II, III and IV IVDs. This indicates that in early stages of IVD degeneration, IHH protein expression in the NP is significantly lower than in the healthy NP and during the end stage IVD degeneration. In addition, decreased *IHH* gene expression was found in cultured single NCs and NC clusters compared with naïve NCD NC-rich NP tissue. It must be mentioned that there is a difference between the *in vitro* cells used for the PCR and the *in vivo* IVD situation used for staining. The cells used for the PCR underwent a cell digestion process prior to culture, which could have affected the expression of (target) genes. Furthermore, the cells used for the PCR do not resemble the whole degeneration process, but only the transition from healthy NCs (present in Thompson grade I IVDs) towards cells with a fibroblast-like phenotype. Thus, for gene expression profiling, the early IVD degeneration process was mimicked *in vitro*. Future studies should focus on *in vitro* researching the degeneration process towards a more degenerated (CLC-like) NP cell phenotype (e.g. representing NP cells from Thompson grade IV-V IVDs). More pronounced *in vitro* IVD degeneration could be achieved by introducing inflammation or mechanical loading in combination with prolonging the culture duration.[3,31]

The current study shows that IHH protein expression increased during IVD degeneration. Previous studies have shown that IHH stimulates cell proliferation and calcification in cartilage.[2,7] The results of the current study may thus indicate that IHH stimulated these processes during IVD degeneration. However, it could also be possible that this increased IHH expression during IVD degeneration is only an incidental correlation instead of a direct causal relationship.

In the cultured NCs, *IHH* gene expression decreased, while gene expression of the receptors *SMO* and *PTCH* increased when compared to the control group (naïve NCD NC-rich NPs). Affirmatively, it is known that when *IHH* gene expression is greatly decreased, more Ptch needs to be expressed to effectively bind this small IHH amount.[32] Moreover, it is known that in the absence of IHH, a transcriptional repressor (Rep-Ci) is generated which binds to Ptch and blocks it's transcription.[21] This could explain why after the initial increased *PTCH* gene expression (T2), PTCH expression was (non-significantly) reduced at T8 and T10 in single NCs.

The *PTHrP* and *PTHR1* gene expression was high at T0 but decreased thereafter. This is probably associated with the very low *IHH* gene expression. IHH stimulates the production of PTHrP and PTHR1, therefore, when the IHH expression decreases, the PTHrP and PTHR1 expression also decreases in this feedback loop.

Study limitations

Although the research did fulfil its aim in regard to IHH protein expression and *IHH* gene expression, some limitations should be noted. The first limitation of this study is that to quantify the percentage of IHH positive cells over the total number of cells, the cells were counted manually (blinded) by one observer. This method is subjective and could be improved by scoring with more than one observer and then take the mean of these scores.

Another limitation that should be taken into consideration is the fact that the NCs were cultured in monolayers. Various studies have shown that monolayer culture influences the cell phenotype.[30,33,34] In a monolayer, the cells proliferate quickly and dedifferentiate. Three-dimensional culture systems, however, promotes their normal morphology and limits proliferation, thus mimicking the *in vivo* behaviour of cells.[30]

Future studies

Future studies are needed to investigate if the transcription factors Gli1, GLi2 and Gli3 are also increased during IVD degeneration. Since this are important downstream signalling molecules stimulated by IHH, it can be used to see if IHH signalling is really affected. Since Sonic Hedgehog (SHH) also uses PTCH as its receptor, it may be useful to investigate if SHH signalling is altered as well during IVD degeneration.

Furthermore, this study only investigated IHH protein expression, but more research could be done to determine the PTHrP, PTHR1, Ptch, Smo and SHH protein expression in the same canine IVDs used for this study. This would give a more complete image of the PTHrP-IHH feedback loop during IVD degeneration.

Another option for future research is to investigate the effect of adding IHH and/or silencing the *IHH* gene on NP cells *in vitro*. A study by Wang *et al*. (2015) showed that siRNA silencing in the vertebral endplate results in a decrease of the transcription factors Gli1, 2, 3 and the cartilage calcification markers Col I and Col X, while the Col II expression increased. These data suggest that siRNA silencing of IHH inhibits degeneration by downregulating the expression of catabolic factors and upregulating the expression of anabolic factors in the cartilaginous endplates.[22]

Lastly, there should be determined whether there is a difference between CD and NCD dogs in IHH expression protein during IVD degeneration, since previous work showed a difference in PTHrP-IHH expression between different breeds.[14]

Conclusion

This study demonstrated a significant positive correlation between IHH protein expression and IVD degeneration grade, which may indicate that IHH stimulates IVD degeneration. Future studies are, however, needed to further elucidate the exact role of the PTHrP-IHH feedback loop in IVD degeneration and to determine if altering this pathway could be a point of engagement for the development of a regenerative treatment.

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