IL1β protein levels in canine intervertebral disc degeneration

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General introduction

Lower back pain is a common problem in humans and our canine companions. Over 80% of the human population suffers from this problem at least once in their lives (Katz, 2006). Associated treatment costs in the United States alone already exceed 100 billion dollars per year in the field of human medicine (Katz, 2006). The underlying cause is not exactly known, but a contributing factor is intervertebral disc (IVD) degeneration, as this causes 40 percent of lower back pain cases in humans (Johnson et al, 2015). The prevalence of IVD degenerative disease in dogs under twelve years of age is generally around 3.5 percent and it is 1.5 times more common in male than female dogs (Bergknut et al, 2010). The associated fatality rate is 1 on 3 (Bergknut et al, 2010). So IVD degeneration and disease are recurring reasons for euthanasia in dogs (Bergknut et al, 2012). All in all, this results in an interesting subject of research for both human- and veterinary medicine.

The healthy IVD

The IVDs transfer loads and provide flexibility, both associated with movement and they make up a third of the spinal cord by height (Bergknut et al, 2013). The IVD develops from the embryonic notochord and mesenchyme (Bach et al, 2014). This development leads to the IVD structure as we know today, consisting of two axial vertebral endplates (EPs), the annulus fibrosus (AF) and nucleus pulposus (NP). The EPs and vascularised parts of the AF play an essential role in providing the IVD with nutrients and oxygen. The AF surrounds the center of the IVD, namely the NP (Bergknut et al, 2013).

The NP consists of a gelatinous matrix, which is mainly made up of proteoglycans and collagen type two. The proteoglycans are aggregated with hyaluronic acid, which results in a negatively charged complex. This negatively charged complex attracts cations, consequently creating an osmotic gradient that attracts water (Bach et al, 2014; Schutgens et al, 2015; Bergknut et al, 2013). In combination with the surrounding AF a hydrostatic pressure is formed in the NP (Schutgens et al, 2015; Bergknut et al, 2013). The matrix of the AF consists of collagen type one, two and proteoglycans. In between the collagen layers, elastin-rich tissue is also present. This provides a firm casing for the NP and prevents bulging of the disc when a load is applied (Schutgens et al, 2015).

In humans and certain dog breeds the young NP contains large vacuolated cells called notochordal cells (NCs) (Figure 1), which are taken over by smaller non-vacuolated nucleus pulposus cells (NPCs) as the IVD matures (Schutgens et al, 2015; Hunter et al, 2004).

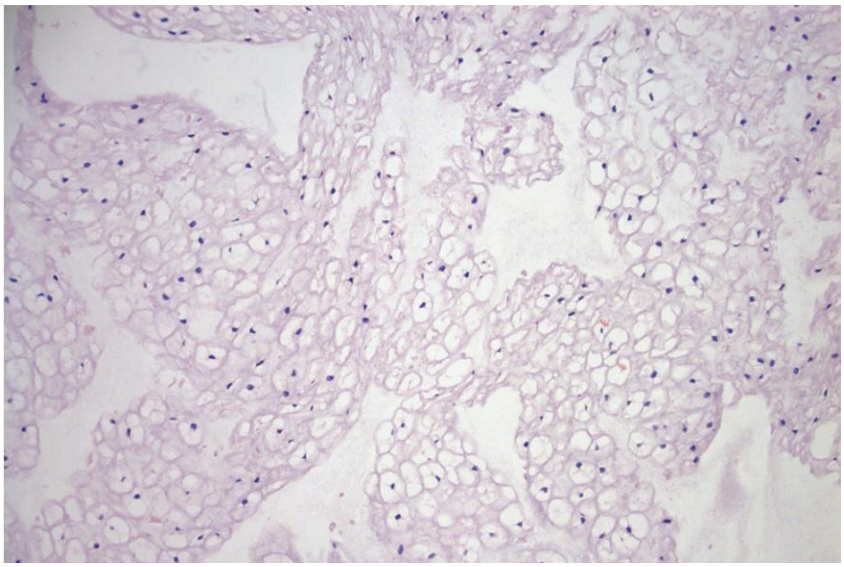

Figure 1. Histology of the healthy IVD
Four times magnified microscopic image of the healthy nucleus pulposus, with clustered notochordal cells. Adapted from Bergknut et al, 2010.

Pathophysiology of IVD degeneration

The exact cause of IVD degeneration is still unknown, but is believed to be multifactorial (Smolders et al, 2012). In chondrodystrophic (CD) breeds it is established that genetics play a significant role (Smolders et al, 2012). More is known regarding the pathophysiology. The swelling and eventually bulging of the disc is caused by the replacement of proteoglycans and collagen type two by fibrous tissue and collagen type one, as can be seen in figure 2 (Schutgens et al, 2015).

The loss of proteoglycans, due to enzymatic activity, results in the inability to maintain the osmotic gradient. Therefore, the IVD loses its attraction to water and becomes dehydrated. This leads to the tearing of the AF and or bulging of the disc, when combined with compressive forces (Bach et al, 2014; Schutgens et al, 2015). This results in a decrease of water content in the NP from 90 percent wet weight to less than 70 percent (Li et al, 2014).

Nerve endings extend further into the AF and NP, whereas normally only the outer third part of the AF is innervated. A nociceptive response can subsequently be triggered by a mechanical or inflammatory stimulus (Willems et al, 2016).

The following noticeable clinical effects are neurological signs and impaired function of the spinal unit. Owners can present veterinarians dogs with unilateral or bilateral lameness, paresis or paralysis. Other symptoms of IVD degeneration and disease can be, for example, difficulties with rising or lying down and movements such as climbing. Once at the veterinarian, pain can also be presented through pressure

during clinical examination. However, IVD degeneration can also be asymptomatic (Bach et al, 2014).

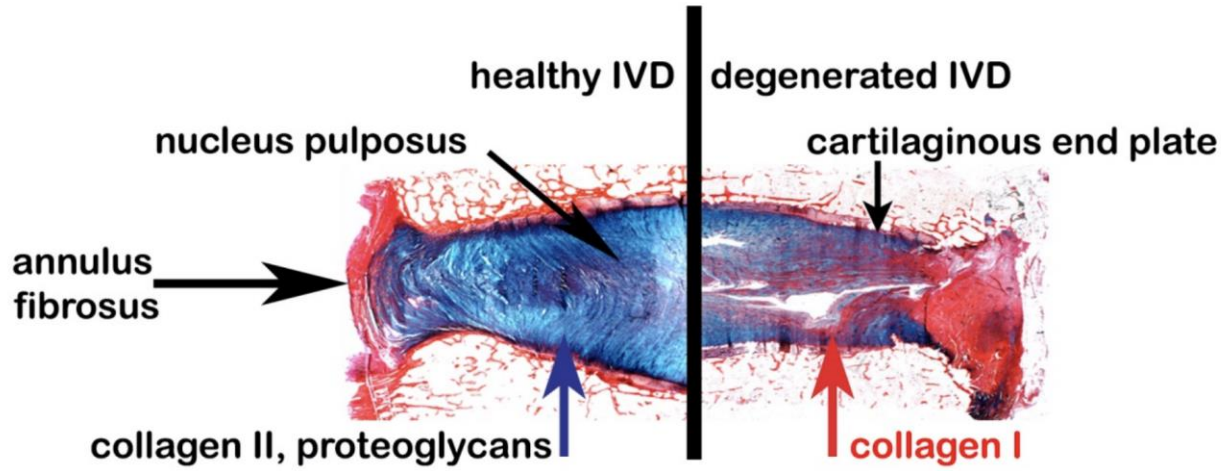

Figure 2. The healthy versus degenerated IVD
The left side shows a healthy human IVD, whilst a degenerated IVD can be seen on the right (Schutgens et al, 2015).

Grading IVD degeneration
The degree of degeneration in the IVD can vary and therefore assessing the right degree can be a valuable tool in order to determine the appropriate treatment strategy and prognosis. Several methods have been approved to be used for this purpose in dogs.

Firstly the Thompson grading scheme. This scheme uses pathological changes of the NP, AF, endplates and periphery of the vertebral body and divides them in five groups. Grade I represents the healthy IVD and grade V represents end stage degeneration (Bergknut et al, 2013b).

Another method that looks at characteristic changes in the IVD structure is the Pfirrmann grading system. Examples of characteristic changes that are analyzed are disc structure, height and ability to distinguish the NP and AF. The surrounding tissue is also taken into account, in order to form a complete picture. However, in order to get more accurate information, the Pfirrmann grading system should be combined with MRI images (Bergknut et al, 2011).

MRI itself is considered a golden standard for diagnostic imaging in IVD degeneration and disease. MRI images allow close analysis of anatomical structures, such as the layers of the IVD (de Costa et al, 2020). The study of Besalti *et al.* (2006) states that IVD disease can be assessed through MRI by four

criteria, which include IVD degeneration, bulging of the disc, disc protrusion and extrusion. However, this has not yet been validated and compared with other standards (Bergknut et al, 2011).

Altogether, MRI, the Pfirrmann grading system and the Thompson grading scheme are important tools in evaluating the state of the IVD.

The role of inflammation during disc degeneration

During the degenerative process, NPCs assemble cytokines such as interleukin (IL)1β and tumor necrosis factor alpha (TNFα). IL1β then enhances IL1β and CXCL8 (IL8) gene expression, which have been associated with lower back pain (de Vries et al, 2019). IL8 is a mediator in the early inflammation process and is a powerful chemokine (Monchaux et al, 2017). These cytokines prompt production of catabolic proteases. An example of such a protease is matrix metalloproteinases (MMP). Inflammatory cytokines are also released by monocytes or macrophages in this inflammatory process (Li et al, 2014).

The quantity of collagen type II decreases as a result of inflammation in the IVD (de Vries et al, 2019). Additionally, IL1β on its own induces proteoglycan breakdown, induces IVD cell apoptosis and inhibits IVD cells from matrix biosynthesis (Li et al, 2014). To summarize, IL1β inhibits IVD maintenance and repair through inhibition of anabolic processes.

The study of Li *et al.* (2014) shows significantly higher IL1β mRNA levels in vitro in NPCs of Beagles where IVD degeneration was surgically induced in comparison to normal NPCs. This seems to confirm the stimulation of IL1β during the degenerative process.

Interestingly, in human IVD degeneration IL1β also exhibits stimulation of angiogenesis (Philips et al, 2015). The study of Lee *et al.* (2011) showed a positive correlation between IL1β expression and vascular endothelial growth factor (VEGF) expression in the degenerated IVD. No information regarding the possibility of a similar phenomenon in canines is known, so it must be kept in mind that there is still much to be elucidated regarding IL1β and its effect in canine IVD degeneration.

All in all, IL1β plays an important role in the degeneration of the IVD and thus lower back pain (Li et al, 2014).

Chondrodystrophic versus non-chondrodystrophic breeds

As stated before, canines are often affected by IVD degeneration. IVD degeneration can occur in all types of dog breeds, however the different breeds can be classified based on the predisposition to chondrodystrophy. Classification can be done in two

distinct groups: chondrodystrophic (CD) versus non-chondrodystrophic (NCD) breeds. Herefrom it could be summarized that CD breeds are more prone to cartilage maldevelopment (Smolders et al, 2012).

CD and NCD dogs differ in the age of onset, frequency and location of degeneration in the spinal cord. Breeds that are included in the CD group are the Dachshund, French and English Bulldog, Cavalier King Charles Spaniel, Welsh Corgi, Beagle, Basset Hound, Shih Tzu, miniature Schnauzer, Pekingese, Lhasa Apso, Bichon Frise, Tibetan Spaniel and American Cocker Spaniel. On the other hand, NCD breeds that often are affected by IVD degeneration are breeds such as the German Shepherd, Dobermann, Rottweiler, Labrador Retriever and Dalmatian (Smolders et al, 2012).

IVD degeneration usually appears around three to seven years of age in CD breeds. In the meanwhile, this development is typically not seen in NCD breeds until six to eight years of age. In addition to this, the NP becomes drier and less gelatinous at three to four months of age in CD dogs (Smolders et al, 2012). At this age point, the NCs in the NP are slowly replaced by a less dense population of smaller non-vacuolated NPCs (Bergknut et al, 2010). In NCD dogs the NCs remain the main cell type during their lives. These cells have a higher rate of apoptosis, which increases over time (Smolders et al, 2012).

The process of chondrification results in a proteoglycan and hyaluronic acid decrease in the NP and an increase in collagen. When it comes to chondrification, endochondral ossification is a point of significant difference between CD and NCD breeds. CD breeds are affected by a disturbed endochondral ossification in the growth plates. As a result, CD dogs have shorter limbs, as the most affected areas are the long bones (Smolders et al, 2012). In addition to this dystrophic calcification, often in the perinuclear area of the NP, is seen in CD dogs. In NCD dogs calcification is normally not seen in the NP (Bergknut et al, 2010).

It has previously been mentioned that proteoglycan levels decrease due to degenerative processes. However, in the NCD breeds the proteoglycan concentration stays more or less at the same level throughout life, whilst a sharp decrease is seen at 30 months of age in CD breeds. In addition to this, chondroitin sulfate side-chains of proteoglycans decrease in the NP of CD breeds and are eventually fully replaced by keratan sulfate. This does too happen in NCD breeds, however at a much slower and gradual rate (Bergknut et al, 2010).

The research of Hansen *et al.* (1952) stated that in NCD breeds the mucoid NP becomes more fibrous, whilst the CD NP changes into a more chondroid structure. This change of NCs to fibrocyte-like cells was not seen in CD breeds (Hansen et al, 1952). However, in more recent research performed by Bergknut *et al.* (2011)

these fibrocyte-like cells appeared to look more like apoptotic NCs. These apoptotic NCs can also be perceived in CD breeds (Bergknut et al, 2010). In CD dogs a strong relationship between age and the level of apoptosis was observed in the research of Klauser *et al.* (2012). Nonetheless, no correlation with the severity of IVD degeneration was found (Klauser et al, 2012). Research performed by Hansen et al (2017) found that chondroid metaplasia is a key part of IVD degeneration in CD and NCD breeds. With chondroid metaplasia the NCs of the NP are replaced by smaller non-vacuolated NPCs (Hansen et al, 2017).

When it comes to location in the spinal cord, IVD degeneration in CD breeds usually occurs in the cervical or thoracolumbar area. The caudal-cervical or lumbosacral portion of the spine is mostly affected in NCD breeds. In addition to this, IVD degeneration more often develops in CD breeds (Smolders et al, 2012). Once IVD degeneration leads to disease, fatality rates of one in five can be found in CD breeds, whilst one in two is the rate found in high risk NCD breeds (Bergknut et al, 2010).

It can be concluded that the degenerative process shows a similar basis in CD and NCD breeds. Nevertheless, differences between these two groups can be found regarding etiology, morphology, histopathology and biochemistry (Bergknut et al, 2010).

The dog as a model for human IVD degeneration

In this study the focus lies on canine IVD degeneration, however research will eventually expand to the human field to form the translation to human medicine and IVD degeneration. The dogs serve as an appropriate animal model, as there are numerous similarities between canine and human IVD degeneration (Bergknut et al, 2012).

When looking at histopathology, glycosaminoglycan (GAG) levels are similar in the IVD degeneration stages of humans and canines (Bergknut et al, 2012). Furthermore, chondroid cell clusters, the disorganized AF and appearing clefts and cracks were found in both humans and canines during IVD degeneration. When it comes to treatment of IVD diseases, medical and surgical procedures are similar in humans and canines (Bergknut et al, 2012).

One of the differences between canine and human IVDs is that the human endplates are significantly thicker and subchondral cortices are thinner. The underlying reason might be that canine growth of the bone takes place in the growth plates whilst humans have no growth plates. In humans this growth takes plates in the junction between vertebrae and endplates (Bergknut et al, 2012). Moreover, the most notable difference is in anatomy, as dogs walk on four legs and

humans on two. It is often believed that the spinal cords of humans endure higher pressure due to gravity. However, research has shown that axial loading patterns in dogs are similar or higher compared to those in humans. When looking at the bone density, this is higher in quadruped animals, indicating higher axial compression stress. It can be concluded that quadruped vertebrae are stronger than human vertebrae (Smit et al, 2002).

When it comes to CD versus NCD breeds as an animal model there are also notable differences. In NCD breeds NCs are still seen in adult IVDs, whilst this is not the case for humans or CD breeds. In CD breeds and humans, NCs are only seen up to one year of age. In addition to this, it is also believed that CD breeds could function as early stage models of IVD degeneration when NCD breeds better display the later onset. Underlying reasoning lies in the etiology, as genetics play a considerable role in IVD degeneration in CD breeds and 'wear and tear' plays an important role in NCD breeds. However, the pathology is similar in both groups (Bergknut et al, 2012).

Other animal models are also used, even though IVD degeneration is often induced in these models instead of occurring spontaneously. In the sand rat, pintail mouse, baboon and dog IVD degeneration occurs spontaneously and therefore they could function as animal models for spontaneous IVD degeneration (Bergknut et al, 2012). All in all, when it comes to determining a canine model for intervertebral disc degeneration chondrodystrophic (CD) breeds such as Beagles are often chosen (Smolders et al, 2012).

Current versus possible future therapies

As of now, common options for treatment of IVD disease are physiotherapy, medication that functions as analgesic or anti-inflammatory and different surgical procedures (Bach et al, 2014). These treatments are mostly symptomatic treatments and do not resolve the underlying issue. However, surgery does reduce compression, as different parts of the IVD can be removed. The underlying cause, namely degeneration, is still not affected by neither of these options. In addition to this, side-effects can occur, such as spinal instability or reappearance of IVD disease (Bach et al, 2014). When it comes to for instance spinal fusion, a known side effect is degeneration of adjacent IVDs. Hence the pain alleviation this procedure brings might not be long term (Schutgens et al, 2015).

The direction science looks towards nowadays is regenerative medicine. Tackling the problem of IVD degeneration at the root would be more beneficial in the long-term and therefore new therapies are developed to repair the degenerated IVD and restore its function (Bach et al, 2014). Possible future therapies include stem cells, gene therapy, growth factors and biomaterial carriers (Schutgens et al, 2015).

Notochordal cells and their matrix

Many studies have been performed on NC conditioned medium (NCCM) as a potential regenerative therapy. This has been a subject of research, as NCCM has shown stimulatory effects on NPCs in in vitro animal models (de Vries et al, 2015). In the study of de Vries *et al.* (2015) NCCM was compared to a base medium (BM). GAG content was doubled in the NCCM group and collagen type two and aggrecan gene expression increased nearly ten-fold in comparison to the BM group. Aforementioned factors and genes are associated with a healthy NP phenotype, which NCCM appears to stimulate (de Vries et al, 2015). This suggests an anabolic and anti-catabolic effect on NPCs, which can possibly be appointed to NCCM (de Vries et al, 2015). Which factors exactly stimulate IVD regeneration is a topic of interest. Previous research has analysed whether soluble or pelletable factors play a key role in the regenerative process. Soluble factors include peptides and proteins, whilst pelletable factors are protein aggregates and extracellular vesicles (Bach et al, 2016). Research done by Bach *et al.* (2016) has stated that NCCM exerts its anabolic effect predominantly through soluble factors in canines and porcines. However, pelletable NCCM factors too showed a moderate level of regenerative effect (Bach et al, 2016).

Often NCCM yielded from porcine tissue is used. This can result in a possible underestimation of the capacities of NCM, as growth factors and cytokines can be species specific (de Vries et al, 2019). When it comes to the species-specific function of NCCM, the research of Bach *et al.* (2015) has shown that porcine and canine NCCM induce NPC matrix production on an equal level. However, considering the cellularity (DNA content) of the NP tissue or protein- or GAG concentration of the NP tissue used for production of NCCM, porcine NCCM seems more potent than canine NCCM. An underlying cause could be the age of the donors, as the porcine donors were multiple months younger than the canine donors (Bach et al, 2015). All species lose NCs in the NP at different ages, even within a species this can vary (de Vries et al, 2019). This makes finding equivalent donors for interspecies analysis difficult. Therefore, the age of the NCCM donor in relation to regenerative effects on degenerated IVDs should be taken into account.

The clinical use of NCCM is in all likelihood not an option, as only small volumes can be injected in the IVD. These small volumes may cause an effect, but this will not lead to a long-term regenerative response (Bach et al, 2016). Therefore, current research focuses on defining the factors in NCCM responsible for the regenerative effects (Bach et al, 2016).

Another example of a promising potential regenerative treatment is the use of healthy porcine derived NC-rich NP matrix (NCM). As already stated before, as the IVD matures NCs are replaced by smaller non-vacuolated NPCs. However, it is

preferable to maintain the NC level as found in healthy IVDs, as NCs secrete matrix rich in proteoglycans and collagen type two. In addition to this, NCs possibly secrete soluble factors that preserve the NP integrity (Bach et al, 2014). NCs have not been found in degenerated IVDs, but NPCs have been found in healthy IVDs. Hence, a loss of the NCs seems to be related to the development of IVD degeneration (Bach et al, 2014). Therefore, sustaining the NC population could function as a regenerative therapy of IVD degeneration due to their potential regenerative role (Bach et al, 2014). As specific factors responsible for the regenerative effects are not known, NCM can be applied. NCM includes the matrix excreted by NCs and therefore challenging identification of the responsible factors can be evaded. In addition to this, NCM has shown significant strong matrix anabolic and proliferative effects (de Vries et al, 2019).

Research of Bach *et al.* (2018) showed that NCM does show anti-inflammatory effects on canine IVDs in vivo. Beagles were subjected to different kinds of treatments in separate parts of the spine. Treatment options included one NCM injection or two NCM injections. Other IVDs received no injections, as they functioned as a control sample. Two injections of NCM resulted in lower PGE2 levels in canine IVDs where nucleotomy (NX) was performed, indicating decreased COX-2 activity. This can be translated as limiting the inflammation process (Bach et al, 2018). In addition to this, downregulated IL1β and TNFα gene expression was found in the IVDs that were treated with two NCM injections (Bach et al, 2018).

Degenerating NPCs produce inflammatory cytokines such as IL1β. In research performed by de Vries *et al.* (2019) IL1β was added to a BM, which resulted in a reduced GAG and DNA content. Subsequent addition of NCM then reduced IL1β gene expression and improved GAG and DNA content. The inflammatory environment did however reduce NCM its anabolic effect, in comparison to the BM. Despite this fact, NCM did reduce IL1β expression significantly, which suggests anti-inflammatory properties and a regenerative potential in the degenerating IVD (de Vries et al, 2019).

NCM as a regenerative therapy has shown strong matrix anabolic and proliferative effects. In this regard, NCM appears to be more potent than NCCM (de Vries et al, 2019). In addition to this, NCM is more easily obtained and available. Therefore its use may be more cost-effective and could evade the process of identifying the active factors in NCCM (de Vries et al, 2019).

Aim

The objective of this study is to (a) optimise the immunohistochemical staining for IL1β in canine IVDs, (b) analyse IL1β protein levels in both CD and NCD dogs of different ages and (c) analyse what the effect of NCM is on the protein level of IL1β in degenerated IVDs in vivo. This will provide insight on the effect of NCM on inflammation in the IVD and thus the potential of NCM to be used as a treatment for IVD degeneration.

Optimising the immunohistochemistry staining protocol for IL1β in the canine nucleus pulposus

Abstract

Immunohistochemical staining is a useful tool when it comes to elucidating the effect of NC-rich NP matrix (NCM) injections on IL1β levels in the intervertebral disc (IVD). Epitope-antibody binding allows visible localisation and quantification of immunopositive IL1β cells. The objective of this part of the study is to optimise the immunohistochemical staining protocol for IL1β in Beagle IVDs. This was done through adjusting different steps of the protocol, such as antibody dilution and antigen retrieval (AR) using citrate buffer. Microscopic images were used to assess the outcome of the adjustments and define what more needs fine tuning. As a first step, an antibody dilution of 0.01 mg/mL with and without antigen retrieval in the form of a 30 minute citrate bath was applied. Results were then used to adapt steps of the protocol. Antibody dilutions of 0.01 mg/mL, 0.02 mg/mL and 0.04 mg/mL all showed some degree of positive staining in the cytoplasm, however positive staining was most evident when using the 0.04 mg/mL dilution. The antigen retrieval with the citrate buffer appeared to have a negative result on the staining. Therefore the antibody dilution of 0.04 mg/mL without antigen retrieval proved to be the best option for staining IL1β in Beagle intervertebral discs, as this resulted in the most distinct and specific staining.

Keywords: Intervertebral disc, Degeneration, Dog, Immunohistochemical staining, IL1β

Introduction

Immunohistochemical staining and influential factors

Immunohistochemical (IHC) staining can be used as a valuable tool for investigating the effect of NCM treatment on the IL1β protein levels. This works through epitope and antibody binding so that specific antigens are elucidated. Through this mechanism the localization and semiquantification of the protein levels of a specific protein can be elucidated.

When it comes to IVD tissue, IHC is the preferred method for detection of specific proteins (Binch et al, 2020). The process of IHC includes incubation with a primary antibody, in order to bind to the specific antigen that is targeted. The second step of antibody incubation makes detection possible, by being mixed with a conjugate called horseradish peroxidase enzyme (HRP) and binding to the primary antibody. The detection becomes visible by using the 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit. HRP converts DAB, which results in the tissue turning a brown color (Binch et al, 2020). Positive staining should be detected in the cytoplasm of the cell, as Pro-IL1β is cleaved in the cytoplasm resulting in IL1β (Soller et al, 2007). Counterstaining with Hematoxylin then makes cellular localization and analysis of the staining possible, as it stains the nucleus and cell wall purple (Binch et al, 2020). Antigen retrieval (AR) can reveal hidden antigens or unmask antigen crosslinking caused by formalin fixation. The options for antigen retrieval are chemical, enzymatic and heat retrieval methods, which all have their own advantages and disadvantages (Binch et al, 2020).

Many steps in this process can be altered to optimize the quality of the staining. Important factors that may be altered are the antibody dilution and or the AR method. By doing this, variations in IHC intensity can be reduced in regard to many antibodies (Kap et al, 2015). Naturally, needed optimization steps depend on the outcome of the first protocol. For example, in case of little or no staining a change of buffer used for primary antibody dilution might be necessary. Ion- or pH changes possibly affect the antibody function and solutions such as PBS or TBS may reduce the sensitivity of the antibody (Goldstein and Watkins, 2008).

The aim of this research is to test and optimize IHC IL1β staining protocols for canine intervertebral discs (IVDs).

Materials and methods

Canine IVDs which were fixated, decalcified and paraffin embedded were used for the IL1β staining. As a positive control sample, canine NPCs cultured in alginate beads with 10 ng/mL IL1β were used. As a negative control sample canine IVDs were incubated with an isotype antibody, in the same dilutions as the IL1β antibody. The slides were deparaffinized by continuous submersion in Xylene (two times for five minutes), 100% Ethanol (two times for three minutes), 96% Ethanol (two times for one minute) and 70% Ethanol (two times for one minute). Hereafter, slides were washed in PBS (once for five minutes), whilst being placed on a platform shaker (Heidolph Duomax 1030 platform shaker). Two to three segments of tissue on each slide were encircled with a PAP pen in order to provide a hydrophobic barrier. Specimens were then incubated with DAKO Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark) for ten minutes at room temperature in order to suppress endogenous peroxidase, pseudoperoxidase and alkaline phosphatase. Slides were washed with PBS (once for five minutes). A part of the slides were then placed in 10 mM citrate buffer in a water bath, the other part was not subjected to an antigen retrieval method. The water bath was preheated at 37 degrees Celsius. Slides were incubated at 70 degrees Celsius for 30 minutes to unmask the antigen. After ten minutes of cooldown time slides were washed in PBS-T 0,1% (two times for five minutes). Hereafter, the slides were washed in PBS-T 0,1% (two times for five minutes) and then blocked in PBS/BSA 5%, whereafter they were left at room temperature for 30 minutes. All slides were then incubated with the primary antibody and left overnight at four degrees Celsius. The mouse monoclonal IL1β antibody (ab156791) was firstly tested in a concentration of 0.01 mg/ML PBS/BSA 5%. As an isotype control Mouse IgG1 (X0931, Dako, Hamburg, Germany) was used in a similar concentration as the primary antibody. The next step included washing the slides in PBS-T 0,1% (two times for five minutes) and consecutively incubating the cells with mouse secondary antibody conjugated with HRP for 30 minutes. Then the slides were washed in PBS (two times for five minutes). The sections were incubated using bright DAB substrate kit (VWRKBS04-110, VWR, Radnor, Pennsylvania, United States of America) for two minutes and then briefly rinsed in demi water. The slides were counterstained by placement in Mayers hematoxylin (Merck 1.09249.0500, Darmstadt, Germany) for one minute. Next, the slides were rinsed in running tap water for fifteen minutes. The slides were then dehydrated through the following consecutive steps, 70% Ethanol (two times for one minute), 96% Ethanol (two times for one minute), 100% Ethanol (two times for three minutes) and Xylene (two times for five minutes). Slides were then coverslipped with pertex.

In the second protocol slides were deparaffinized, washed in PBS and blocked with DAKO enzyme block as stated in the first protocol. The steps after the incubation with primary antibodies differ from the first protocol. The mouse monoclonal IL1β

antibody was diluted to 0.04 mg/ml and mouse IgG was used as a negative control. The following steps matched the first protocol and included washing in PBS-T 0,1%, incubation with mouse secondary antibody conjugated with HRP, washing in PBS, incubation in bright DAB, brief rinse in demi water, Mayer hematoxylin (Merck 1.09249.0500, Darmstadt, Germany) as counterstain, rinsing in running tap water, dehydration steps and coverslips with pertex.

In the subsequent protocol 0.02 mg/mL and 0.04 mg/mL dilutions of the primary antibody were tested without AR. The other steps of the protocol were corresponding with the steps mentioned above.

Acquisition of microscopic images

Images of the sections were taken with the Olympus BX43 light microscope. 10 and 20 time magnification were used to evaluate the staining.

Results

Microscopic images made testing out the different protocols can be found in figure 3. The first row shows the results of the isotype control and IL1β staining in the positive control. As a negative control a Beagle IVD was used, which was incubated with the isotype control antibody. Alginate beads cultured with IL1β functioned as a positive control. No specific staining was found in the negative control, whereas specific cytoplasmic staining in the positive control was detected. The second row shows the results of the first protocol, where 30 minutes in citrate versus no AR was tested with an antibody dilution of 0.01 mg/mL. The third row shows 0.02 mg/mL versus 0.04 mg/mL antibody dilution without AR. Evident positive staining (indicated by the arrowheads) is visible in canine IVDs when no AR and antibody dilutions of 0.02 mg/mL or 0.04 mg/mL are applied. However, 0.04 mg/mL dilution shows the most distinct staining.

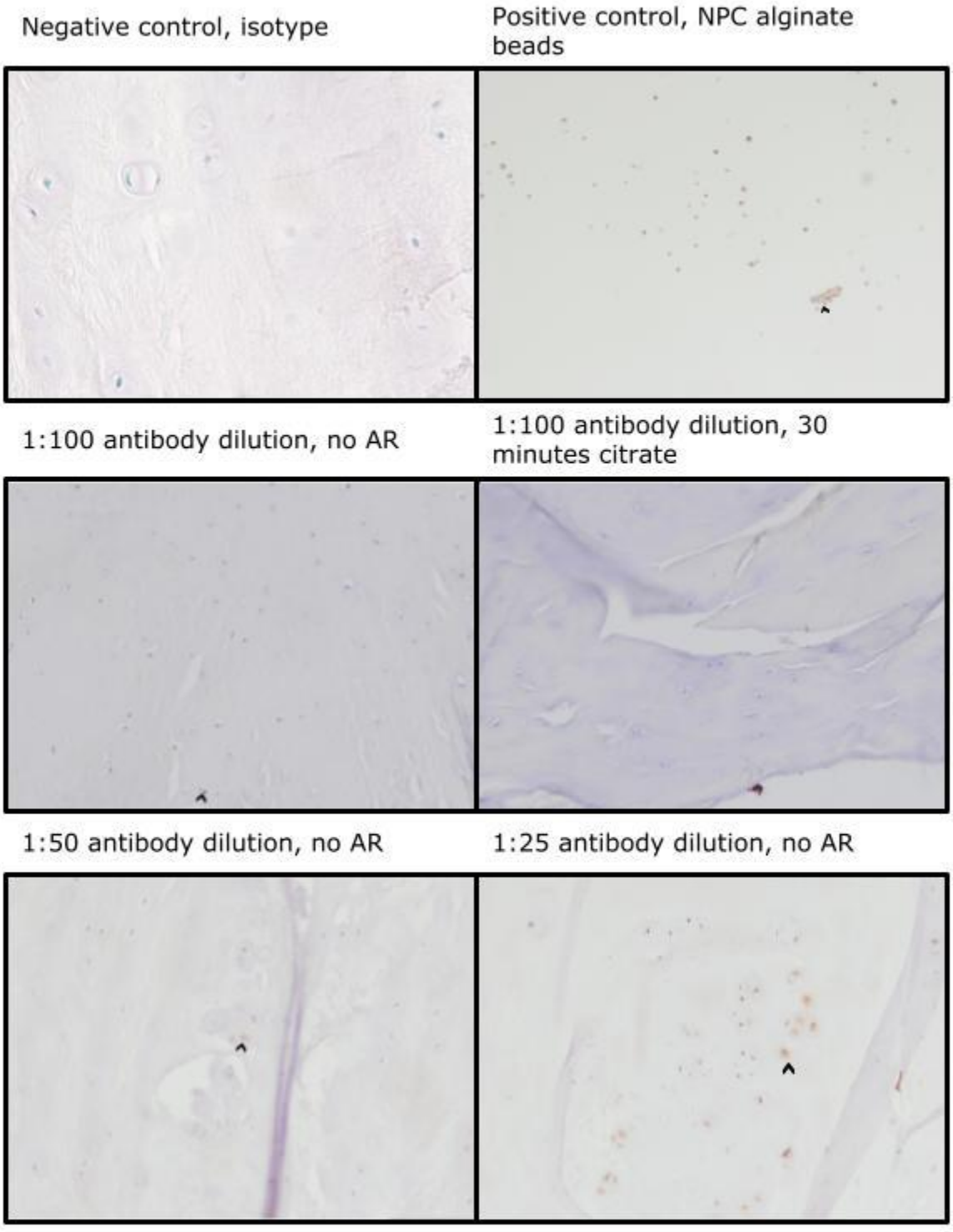

Figure 3. Microscopic images after protocol adjustments
10 time magnified microscopic images of canine NPs stained with different protocols for IL1β. Arrowheads show examples of positively stained NPCs.

Conclusion

This study investigated which protocol was optimal for the IHC staining of IL1β in canine IVDs. Crucial steps that varied between protocols were AR versus no AR and antibody dilution. For canine IVDs no AR and an antibody dilution of 0.04 mg/mL were proven to be the most optimal protocol, as this provided evident specific cytoplasmic staining. 0.01 mg/mL antibody dilution showed less specific staining than the 0.02 mg/mL dilution. However, in both cases the apparent visible staining was less distinct than when the 0.04 mg/mL dilution was applied. Therefore, in continuation of the research no AR and 0.04 mg/mL antibody dilution were used as a protocol.

IL1β protein levels in CD versus NCD breed intervertebral discs

Abstract

Intervertebral disc (IVD) degeneration in dogs can be classified in a chondrodystrophic (CD) or non-chondrodystrophic (NCD) group, due to their predisposition to IVD degeneration. Besides their many differences, such as the occurrence and location of IVD degeneration, the fundamentals of the pathological processes leading to degeneration are similar. More information regarding the role of inflammation in the two groups is missing and therefore the aim of this study is to analyse the IL1β protein levels in IVDs of NCD puppies, adult NCD- and CD dogs. CD dogs were expected to have higher IL1β protein levels in the NP, as they generally have a higher level of IVD degeneration. In order to obtain results, NPs of two stillborn labrador puppies, two adult NCD- and two CD dogs were fixated and immunohistochemically stained for IL1β. The results showed a general trend of low IL1β protein levels in the NP of puppies, higher levels in NCD dogs and the highest in CD dogs. Immunopositive cell rate values were found to be 0.025, 0.15 and 0.25 respectively. This fits the expectation of a possible link between IL1β protein levels and the grade of degeneration, as the least degeneration is expected in puppies and the most in CD dogs. However, no definitive conclusions can be made as high donor variation was present and a bigger donor group is needed. In addition to this, degeneration was not previously assessed and therefore no statements regarding the grade of degeneration can be made. Future research can focus on expanding the donor group and analysing levels of other cytokines related to IVD degeneration. This will prove to be fruitful, as more information on the differences and similarities between the two groups can provide further insight in the steps towards the development of new treatment strategies and translational medicine.

Introduction

In canines intervertebral disc (IVD) degeneration can be classified in a chondrodystrophic (CD) or non-chondrodystrophic (NCD) group, due to the predisposition to chondrodystrophy. Differences between the two are age of onset, frequency and location of degeneration (Smolders et al, 2012).

IVD degeneration can already occur at three years of age in CD breeds and the degenerative process where the nucleus pulposus (NP) becomes drier can start at three months of age (Smolders et al, 2012). In NCD breeds the process of degeneration occurs much more gradually at six to eight years of age. Proteoglycan levels and the presence of notochordal cells (NC) in the NP stay more or less at the same level throughout life, whilst both sharply decrease in CD breeds (Bergknut et al, 2010). In addition to this, in NCD breeds IVD degeneration usually occurs in the caudal-cervical or lumbosacral region due to 'wear and tear', whilst genes seem to play an important role in the etiology of cervical or thoracolumbar IVD degeneration in CD breeds (Smolders et al, 2012).

Research has shown that beside these differences, a similar basis in the degenerative process is seen in both types of breeds (Berknut, 2011). The replacement of NCs in the NP by smaller non-vacuolated nucleus pulposus cells (NPCs), chondroid metaplasia, can be seen in both CD and NCD breeds. Therefore, chondroid metaplasia can be seen as a key part of IVD degeneration (Hansen et al, 2017).

It has previously been stated that NCD and CD breeds show differences regarding proteoglycan and collagen content in IVD degeneration (Bergknut et al, 2010). However, not much is not about inflammation in the two groups. Analysis of which cytokines partake in IVD degeneration in NCD and CD breeds and to what extent, could give some further insight into the differences and similarities between the two. Therefore the aim of this part of the study is to analyse the IL1β levels in NCD dogs (puppies and adults) and CD dogs. CD dogs are expected to have higher IL1β protein levels as IL1β could play a part in IVD degeneration (Smolders et al, 2012; Li et al, 2014).

Materials and method

The aim of this research was to determine IL1β protein levels in CD and NCD breeds and over the course of life. To do so the NPs of six donors were analysed. Two NPs were obtained from stillborn labrador puppies, two from adult mongrels and two from adult beagles. Labradors and mongrels qualify as NCD breeds, whilst beagles are CD breeds. The exact age of the donors can be found in table 1. The mongrels and beagles were sacrificed for unrelated studies and the IVDs were collected. After decalcification and fixation the IVDs were embedded in paraffin and 5 µm sections were placed on slides.

IL1β immunohistochemical staining and analysis

The staining protocol selected in the first part of this study, with no antigen retrieval (AR) and 0.04 mg/mL antibody dilution, was also used in this part of the study. Previously stated information regarding immunohistochemical staining applies here as well, positive staining with IL1β was defined as orange-brown staining in cytoplasm and the positive cell ratio was calculated.

Breed	Age	Sex
Labrador 1 (NCD breed)	Stillborn	Unknown
Labrador 2 (NCD breed)	Stillborn	Unknown
Mongrel 1 (NCD breed)	1 year	Female
Mongrel 2 (NCD breed)	1 year	Female
Beagle 1 (CD breed)	4 years	Female
Beagle 2 (CD breed)	3.5 years	Female

Table 1. Donors

Results

Figure 4 shows the microscopic images of the immunohistochemical staining of IL1β in the NPs of NCD puppies, NCD adult dogs and CD dogs. Examples of positive cells are indicated with arrowheads and positive cells were manually counted for quantification. The results herefrom are shown in figure 5. The puppies showed generally very low IL1β levels, as the mean ratio of immunopositive cells was 0.025. In NCD and CD dogs this was higher, as the mean was 0.15 and 0.3 respectively. The donor variation between the two puppies appeared to be 0.05, whilst this was 0.1 for the two NCD dogs and 0.065 between the two CD dogs. The general trend illustrated in figure 5 is that NCD puppies show the lowest IL1β levels and CD dogs the highest, IL1β levels of NCD can be found more or less in the middle of the two other groups.

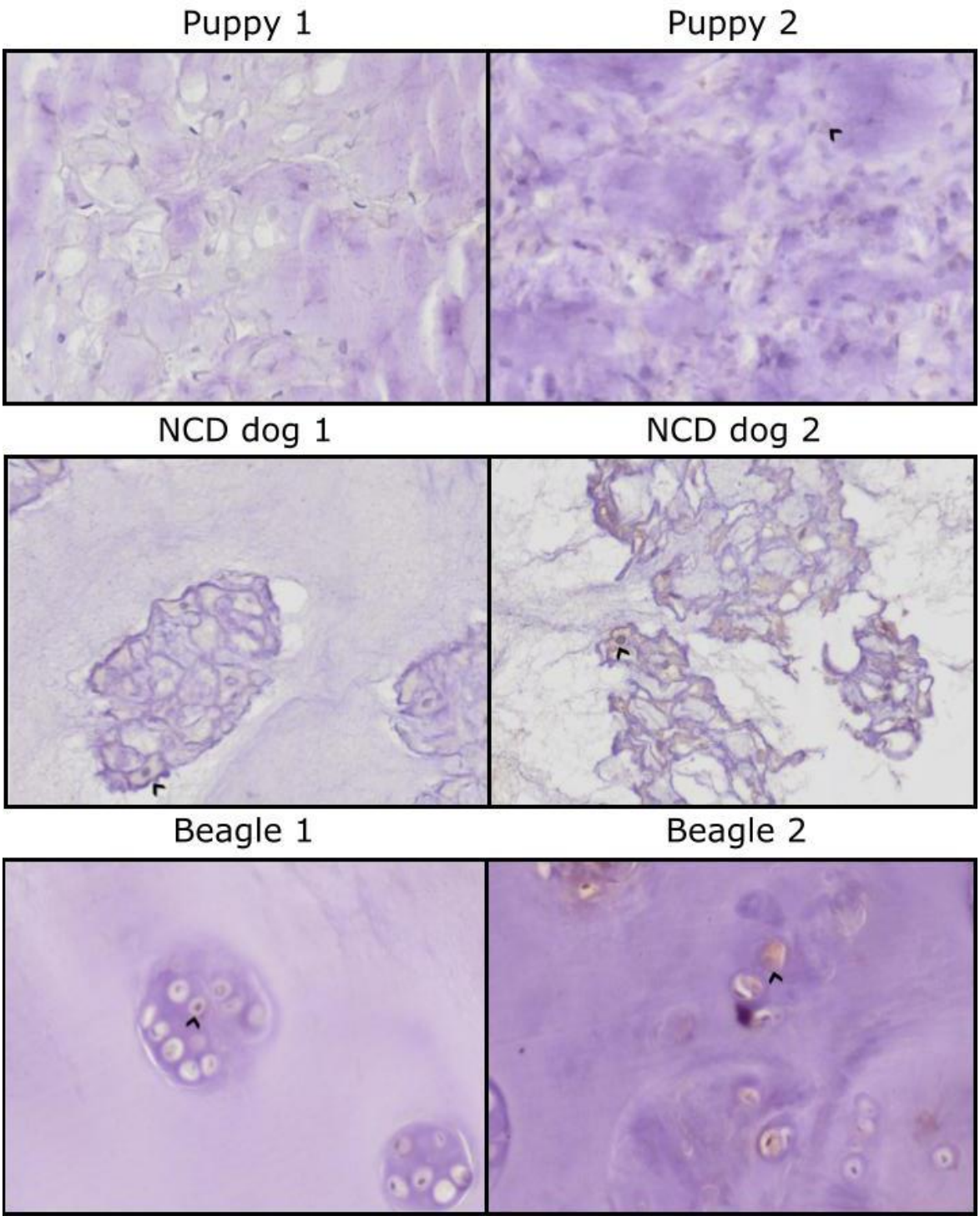

Figure 4. Immunohistochemical staining of IL1β in NCD puppies, adult dogs and CD adult dogs

Twenty times magnified microscopic images of canine NPs of puppies, NCD dogs and CD dogs, stained for IL1β. Arrowheads show examples of positively stained NPCs.

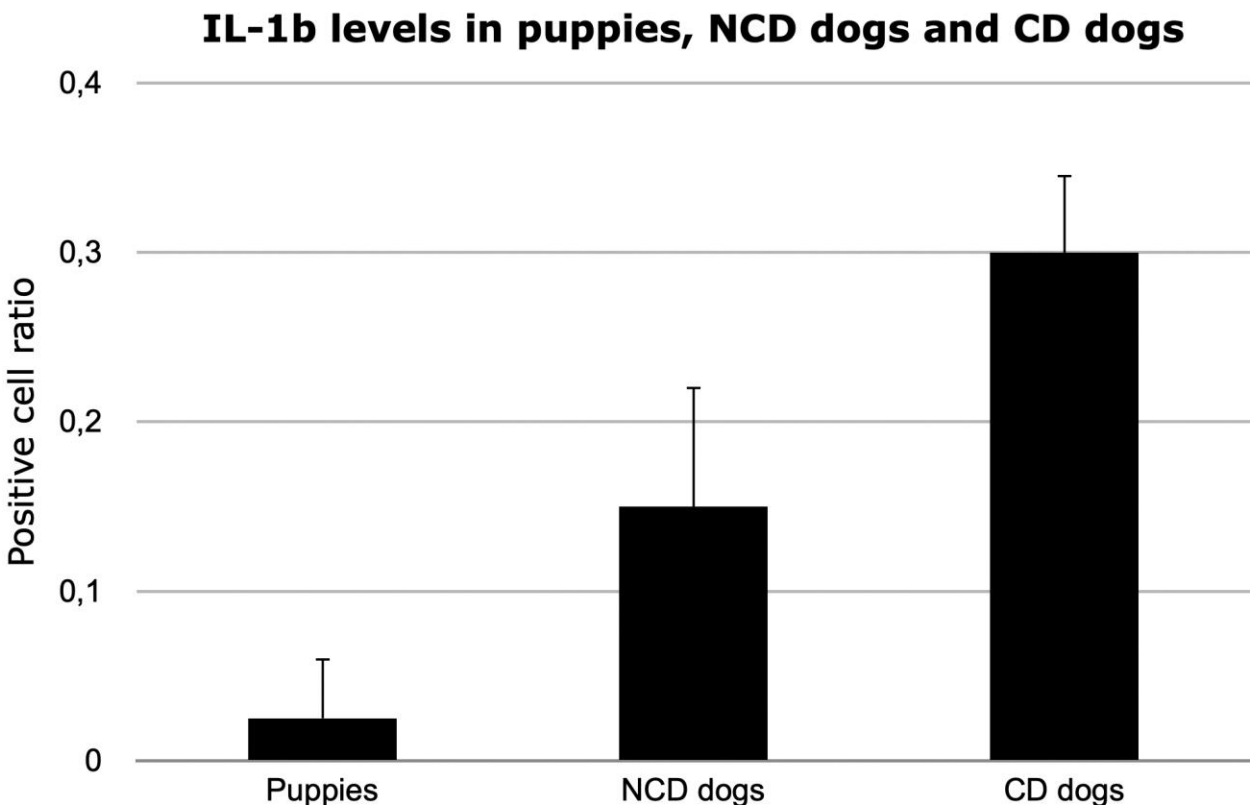

Figure 5. Immunopositive cell ratio of IL1β in the different donors

Discussion

Aim

This part of the study aimed to determine and analyse IL1β protein levels in the IVDs of CD and NCD breeds, as not much is known regarding cytokine levels in the two groups. To do so, NPs of two labrador puppies, two adult NCD and CD dogs were obtained. The puppies were stillborn and other donors were sacrificed for unrelated experiments. After decalcification and fixation the NPs were embedded in paraffin and placed on slides. The NP sections were then immunohistochemically stained for IL1β.

Higher IL1β protein levels in the NPs of CD breeds

The general trend seen in the results was that IL1β protein levels were low in NCD puppies, higher in adult NCD dogs and highest in CD dogs. With differences between the mean ratio of immunopositive cells ranging between 0.1 and 0.15. IL1β can be used as a marker for inflammation and possibly corresponds with IVD degeneration. As stated earlier, IL1β induces proteoglycan breakdown, IVD cell apoptosis and inhibits matrix biosynthesis (Li et al, 2014). Therefore it can be concluded that IL1β stimulates matrix breakdown and IVD degeneration. A previous study in this research group found that herniated NPs of CD dogs show a higher PGE2-weight ratio in comparison to herniated NCD samples (thesis S. van Dongen, 2014). This was in accordance with expectations and results of this study. Expectations were that the NPs of NCD puppy IVDs showed little to no degeneration and that CD dogs show a higher level of degeneration than puppy and adult NCD dogs. Therefore, the levels detected in the present study fit the hypothesis.

Limitations and future studies

One limitation of the study was the amount of donors, since there were only two donors included per group. Furthermore, the donor variation in IL1β protein levels within the different groups was apparent. A general trend was distinguishable between the groups, but the immunopositive cell rate did differ between donors within the same group. To overcome this, it would be preferred to expand the donor group in future research. Once the results are reciprocated with more donors, more definitive conclusions could be drawn. In addition to this, in order to draw conclusions about IL1β levels corresponding with degeneration, it would be good to determine the grade of degeneration in IVDs that are used for quantifying IL1β levels. This can be done through, for example, an MRI in the live animal, macroscopic grading (Thompson grade) or histologic changes in the post-mortem spine. In the latter case, a hematoxylin and eosin and Safranin O Fast Green staining could be applied in order to look at cell morphology and GAG levels, which would obtain an indication of the state of the NP (Bergknut et al, 2013b). Although all the donors within the groups were more or less in the same age range, the adult NCD dogs were two to three years younger in comparison to the CD dogs. This

difference in age hampers proper comparison between the two groups. IVD degeneration is known to develop during lifetime (Smolders et al, 2012) and thus age of the donor should be taken into account. Therefore, future studies should analyse IL1β protein levels in NCD and CD breeds of similar age and gender. Next to that, analysing IL1β protein levels at different points of life could provide more insight into possible changes related with aging. Not only protein levels, but also measuring IL1β expression through PCR can provide interesting results. In addition to this, different genders or breeds can be studied as there might be gender- or breed related differences, which could lead to interesting results. Also looking into other cytokines related to inflammation, could further elucidate the pathology of IVD degeneration in two groups and could provide more insight into this subject. Possible cytokines that can be studied are IL6 and IL8, as they are produced by degenerating NPCs and have been linked to lower back pain (Burke et al, 2002; de Vries et al, 2019).

Degenerating NPCs produce inflammatory cytokines such as interleukin (IL)-1b and tumor necrosis factor a (TNFa).4,5 IL-6 and IL-8 produced by degenerating NPCs have been linked to low-back pain.

Another limitation was the lacking information regarding from which regions of the spines the NPs were collected. NCD and CD breeds demonstrate IVD degeneration most prominently in certain regions of the spine (Smolders et al, 2012) and therefore, this plays an important role in the interpretation of the results

Lastly, cell counts were performed manually, which can result in a subjective error. To refrain as much as possible from this, cell counts were performed by one person and then checked and evaluated by a second person. In addition to this, the cell counts were done on two twenty time magnified microscopic images, which covers only a limited part of the NP. In order to provide a more representative cell count, additional microscopic fields should be included in the cell count.

Conclusion

In this part of the study IL1β levels were analysed in NCD puppy, adult NCD and CD dog NPs. IL1β levels were the lowest in NCD puppies and the highest in CD dogs. This was in line with our expectations, as the least degeneration is expected in the NCD puppy IVD and the most in the CD dog IVD, due to their predisposition to degeneration. Our results therefore suggest that IL1β levels increase during IVD degeneration. For future research it would be interesting to repeat this study with more donors of different genders and ages. This will be necessary in order to draw more concrete conclusions, as the donor pool used in this study was relatively small. In addition to this, an MRI analysis of the donors would be useful in order to provide conclusions in regards to the possible correspondence between IL1β protein levels and the grade of IVD degeneration. Further delineating the role of IL1β in IVD degeneration in different breeds and life stages could be beneficial for developing new treatments for lower back pain due to IVD degeneration in dogs and the translation to human medicine.

The effect of NCM on IL1β protein levels in degenerated Beagle IVDs

Abstract

Background: One of the underlying causes of lower back pain is intervertebral disc (IVD) degeneration, where replacement of proteoglycans and collagen type two results in swelling and bulging of the disc. Present-day treatment focuses on tackling symptoms or, temporarily, alleviating pain through surgical procedures. However, current research looks at the field of regenerative medicine. Therapeutic strategies such as notochordal cell-conditioned medium (NCCM) and notochordal cell-rich nucleus pulposus matrix (NCM) have shown a regenerative effect on the NP. This study builds on the research of Bach et al (2018), which has shown the anti-inflammatory properties of NCM, as inflammation plays a role in IVD degeneration. The effect NCM has on IL1β protein levels has not been researched yet and is therefore the aim of this study. We hypothesize that NCM injections decrease IL1β protein levels in the NP. This study focused on analysing the effect of NCM injections on IL1β levels in mildly and moderately degenerated Beagle IVDs, by use of the protocol that was optimized in the first part of this thesis. Overall, the IL1β expression was higher in the NPs of moderately degenerated IVDs compared with the mildly degenerated IVDs. No effect of the NCM injections on IL1β protein levels were detected in the NPs of the mildly degenerated IVDs. Unexpectedly, the NPs of the moderately degenerated IVDs showed an increase in IL1β levels after NCM injections, although not significant. Additional research is necessary to find out whether this increase in IL1β by NCM could be considered anabolic or catabolic.

Keywords: Intervertebral disc, Degeneration, Dog, Notochordal-cell matrix, IL1β, Regenerative medicine

Introduction

NC-rich NP matrix (NCM) appears to be a possible regenerative therapy with a lot of potential, as maintaining the notochordal cell (NC) level in the nucleus pulposus (NP) seems to be associated with preserving the integrity of the NP (Bach et al, 2014). NCM has shown strong matrix anabolic and proliferative effects on bovine nucleus pulposus cells (NPCs) in vitro. In addition to this, NCM is easily obtained and available (de Vries et al, 2019). This study builds on the research of Bach *et al.* (2018), in which they had shown that NCM has anti-inflammatory effects on canine intervertebral discs (IVDs) in vivo. In this study, decreased PGE2 protein levels and gene expression of IL1β and TNFα were detected. Which effect NCM has on IL1β protein levels has not been studied yet.

The previous part of this thesis looked at IL1β protein levels in non-chondrodystrophic (NCD) puppies and adult dogs and chondrodystrophic (CD) dogs. This resulted in the detection of higher IL1β protein levels in the NPs of CD breeds when compared to the NPs of NCD breeds, both in puppies and adult dogs. These results are also interesting for the present study, since NC-rich NPs in the NCD breeds appeared to have lower IL1β protein levels and donors used in this part of the study received the NC-rich NP matrix. In addition to this, CD breeds usually have a higher level of degeneration at a young age, so it would be interesting to see if IL1β protein levels correlate with the degree of degeneration and if NCM would be able to halt or even decrease the inflammatory process.

The aim of this research was to analyze if, and to which extent, NCM injections affect IL1β protein levels in the degenerated canine IVDs. Based upon aforementioned research and the results of the previous part of this thesis, we expected that NCM injections decrease IL1β protein levels in the NP. This will be assessed by immunohistochemical staining for IL1β, using the optimized protocol determined in part one of this thesis.

Materials and methods

Study design and population

The aim of this research was to determine the effect of NCM on IL1β levels in degenerated canine IVDs and builds on the latest work of Bach *et al.* (2018). The IVDs of five intact female Beagles were analyzed. All five were fourteen months of age, with weight ranging between ten to eleven kilograms. The NCM was derived from NC-rich porcine tissue and obtained through lyophilization, pulverization and resuspension at 10 mg/mL. IVD degeneration occurred spontaneously or was induced by partial NP removal (NX) on the left side of the spine. The spontaneous IVD degeneration was considered mild and the induced moderate. Six weeks before these intradiscal injections the dogs were clinically examined by veterinarians, which included an orthopedic examination in addition to the general checkup. Furthermore, MRI analysis took place before the injections, to determine the degree of IVD degeneration. Non-randomized treatment options were applied, in order to prevent random effects that would interfere in the research. IVDs both with and without NX served as a control (no injection) or were injected in the right side with NCM (50 µL). Three months after the (first) injection MRI analysis was performed and two IVDs per dog received a second NCM injection (50 µL), to determine whether multiple injections yielded a greater effect. The location where the donors received the injections can be seen in table 2. Six months after the first injection MRI and CT analysis took place, whereafter the dogs were euthanised and the IVDs were collected. Analysis of the results was performed by blinded investigators (Bach et al, 2018). After decalcification and fixation the IVDs were embedded in paraffin and 5 µm sections were placed on slides.

IL1β immunohistochemical staining and analysis

The sections of the above mentioned study were stained using the aforementioned protocol and analyzed to determine the protein levels of IL1β in the NPs of the IVDs subjected to different treatments. Positive staining with IL1β was defined as orange-brown staining in cytoplasm (Uniprot, n.d.). For the immunopositive cell ratio, three random areas were photographed in twenty times magnification. Hereafter, two areas were chosen and counted manually and the positive cell ratio was calculated.

For the statistical analysis, IBM SPSS statistics 26 was used. A Shapiro Wilks test was applied to check if the data was normally distributed or not. As the data were not normally distributed, a Kruskal Wallis and Mann-Whitney U test were performed. P-values of <0.05 after correction were considered to be significant.

Location	Treatment	Dog	Location	Treatment	Dog
T11-T12 L1-L2	Control	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5	L6-L7 L7-S1	2x NCM	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5
L1-L2 T11-T12	NX Control	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5	L7-S1 L6-L7	NX 2x NCM	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5
L2-L3 L3-L4	1x NCM	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5			
L3-L4 L2-L3	NX 1x NCM	Dog 1 Dog 2 Dog 3 Dog 4 Dog 5			

Table 2. Donors and treatment

Results

NCM treatments in IVDs without partial nucleotomy

Figure 6 shows the microscopic images taken of the NP of the IVDs that were not subjected to NX (mildly degenerated). Arrowheads show examples of immunopositive cells. Immunopositive cells were counted for quantification. Results of quantification can be seen in figure 8. In dog one, one NCM injection appeared to have no effect, but two injections lowered the IL1β protein levels. In dog two, one and two NCM injections decreased IL1β protein levels to the same level. In dog three no IL1β was detected after one NCM injection, however two NCM injections brought the IL1β protein levels back to the level as seen in the control NP. In dog four a higher level of IL1β was seen after two NCM injections, whereas no IL1β was seen after one injection. In dog five a higher IL1β protein level was seen after one NCM injection in comparison to two NCM injections. In the control NP of dog four and five no IL1β was detected, whereas dog two had the highest protein level of IL1β in the control IVD. In general, there was no clear general trend detected between the different treatment groups.

NCM treatments in IVDs with partial nucleotomy

Figure 7 shows the microscopic images taken of the NP tissue of the IVDs that underwent partial nucleotomy (moderately degenerated). Arrowheads show examples of immunopositive cells. Immunopositive cells were counted for quantification. Results of quantification are also shown in figure 8. As expected, the IL1β expression was higher in the NPs of the moderately degenerated IVDs compared with the expression in the mildly degenerated IVDs in all treatment groups. In three of the donors (dog one, two and five) there was an increasing trend detected in the IL1β protein expression in the NP when injected with NCM, although not significant. In dog one there was a bigger difference between the IL1β level with one or two NCM injections than there was in dogs two and five. In dog two, relatively higher IL1β protein levels were seen in all three conditions, in comparison with the IL1β levels in the other donors. In dog three, a higher IL1β protein level was seen regarding the one time NCM injection, however no IL1β was detected after two NCM injections. In dog four a lower level of IL1β was seen after one NCM injection, but a higher level was seen after two NCM injections. IL1β protein levels in dog five appeared to increase with each NCM injection. When it comes to the mean trend of all donors, increasing IL1β protein levels were seen with each NCM injection.

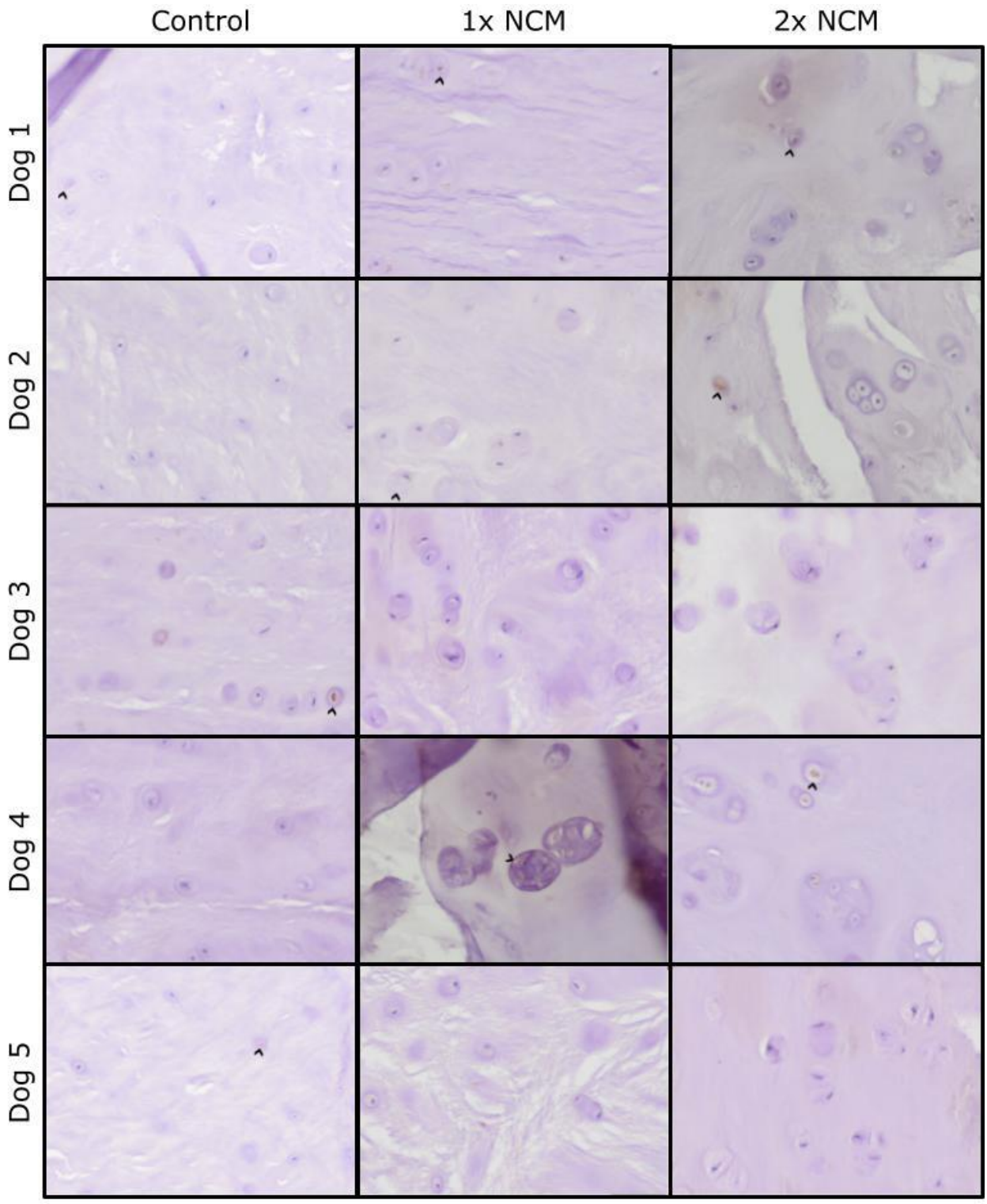

Figure 6. No-NX samples immunohistochemically stained for IL1β

Twenty times magnified microscopic images of canine NPs which have not undergone partial nucleotomy (NX), stained for IL1β. Arrowheads show examples of positively stained NPCs.

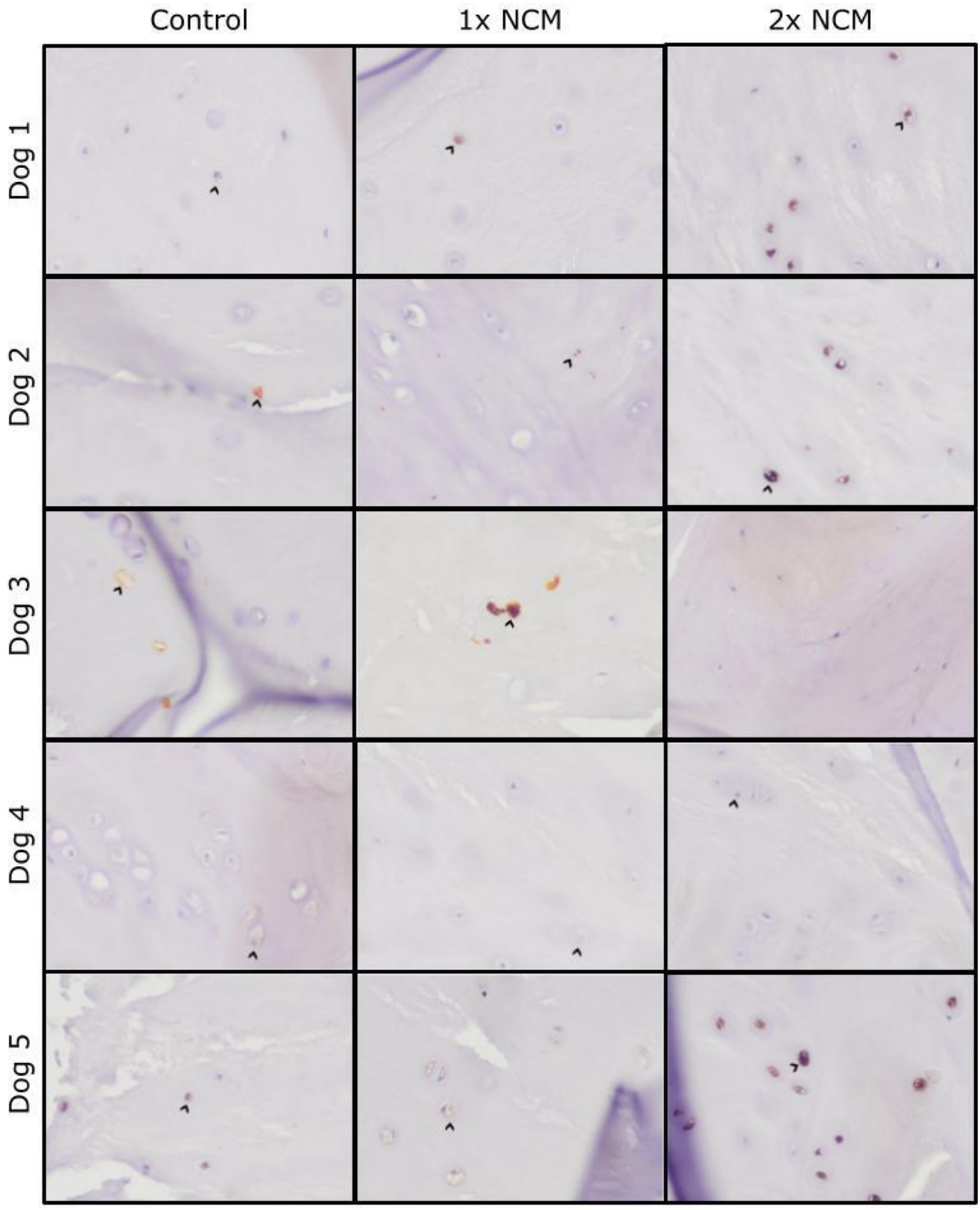

Figure 7. NX samples immunohistochemically stained for IL1β

Twenty times magnified microscopic images of canine NPs which have undergone partial nucleotomy (NX), stained for IL1β. Arrowheads show examples of positively stained NPCs.

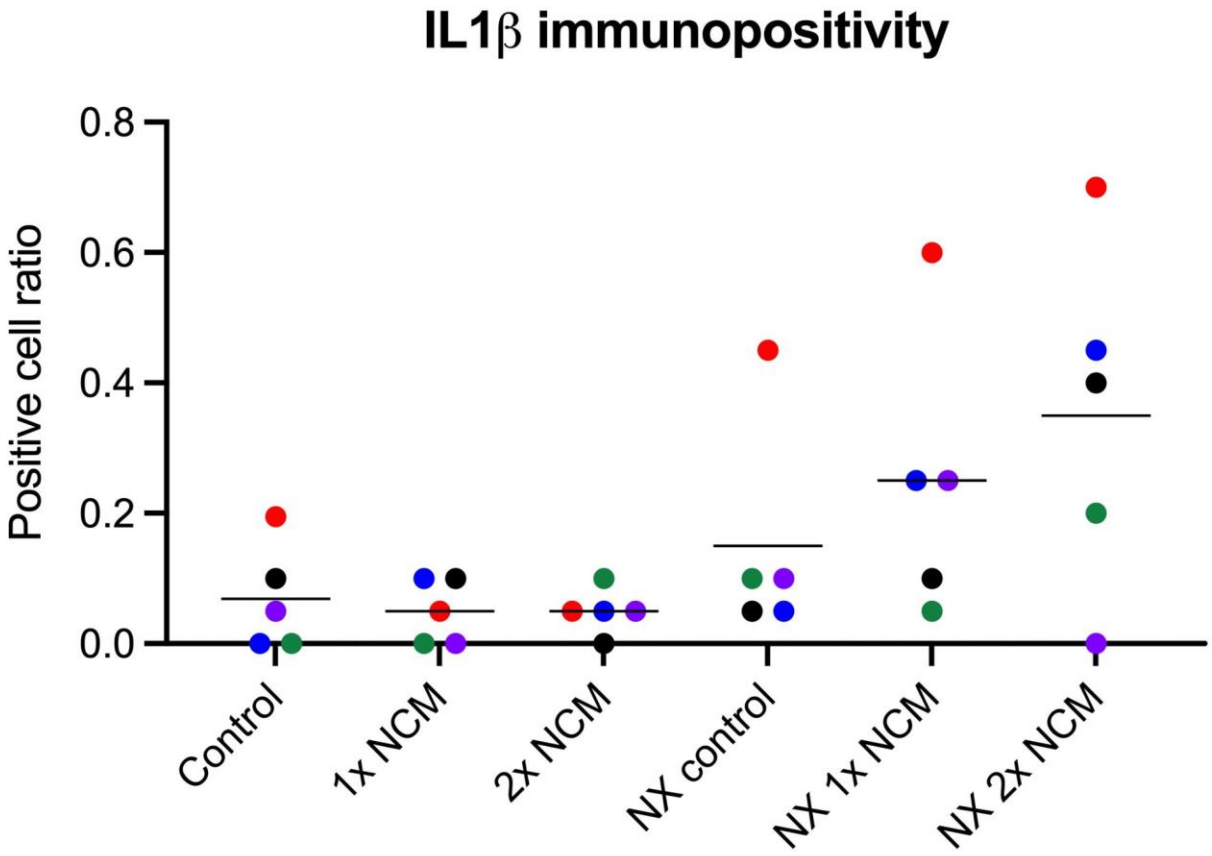

Figure 8. Graph of positive cell ratio in the different donors.

Legenda
Dog 1 = Black
Dog 2 = Red
Dog 3 = Purple
Dog 4 = Green
Dog 5 = Blue

Discussion

Aim

This study builds on the research done by Bach *et al.* (2018). In this *in vivo* study both mildly and moderately degenerated Beagle IVDs were subjected to no treatment (control) or one or two times injection with NCM. Their results indicated an anti-inflammatory effect by NCM by decreased protein level of PGE2 and gene expression levels of IL1β and TNFα. To confirm this effect on IL1β protein level, in this study immunohistochemical staining of this key cytokine was performed on the same IVD samples.

Comparison with previous studies

The *in vivo* research of Bach *et al.* (2018) showed that IL1β and TNFα gene expression did not differ between no-NX and NX control samples. However, PGE2 levels were higher in control NX IVDs in comparison to control no-NX IVDs. According to their results, the two NCM injections did result in a reduction of IL1β and TNFα expression and PGE2 content in NX IVDs. These results imply that two NCM injections decrease COX-2 activity and inhibit inflammation (Bach et al, 2018). In accordance with the research performed by Bach *et al.* (2018), this study also showed generally higher IL1β protein levels in NX control IVDs in comparison with no-NX IVDs. Indicating that IL1β protein levels increase with degeneration state. In addition to this, both studies show quite some donor variation. In both cases no specific correspondence could be detected between the donors and how they responded to the different treatment options.

In contrast to these similarities, the results of the present study differ when it comes to the effect of two NCM injections in NX IVDs. Where the research of Bach *et al.* (2018) showed a decrease in IL1β gene expression, this study actually showed an increase in IL1β protein levels. Often an assumption is made that mRNA expression implies corresponding changes in protein levels (de Sousa et al, 2009). However, multiple studies in the human field have stated a poor correlation between the two, as only 40 percent of protein levels can be explained by mRNA expression. Several biological factors can have an effect on this correlation and specific drug-treatment can also interfere for example (Koussounadis et al, 2015). Further analysing IL1β gene expression and protein levels could give more specific information about the effect of NCM on translation and degradation of IL1β (Koussounadis et al, 2015).

Higher IL1β protein levels with NCM injections in NX NPs

Earlier research resulted in the hypothesis that NCM injections would decrease IL1β protein levels. Surprisingly, especially in the NX IVDs, IL1β protein levels seem to progressively increase with each injection. A possible cause could be the intradiscal injections themselves. The tip of the needle passes through the AF into the NP and

could therefore cause slight damage resulting in higher IL1β levels. In the study of Willems *et al.* (2015) a 26G needle was used to inject 40 microliter, which did not result in degenerative changes in the IVD, however nothing is particularly stated about IL1β levels and inflammation. It should be taken into account that once the needle diameter to disc height ratio exceeds 40 percent, IVD degeneration has been observed (Elliott et al, 2008). Another factor to be mindful of is that the NCM used was derived from porcine tissue and injected in canine IVDs. In the study of Tsai *et al.* (2014) injection of porcine adipose-derived stem cells in the canine stifle joint did not result in an inflammatory or allergic reaction. However, similar research has not yet been performed regarding porcine derived NCM injected in canine IVDs. In some cases a single injection would not lower IL1β levels, but two injections would. The underlying reason could possibly be the admitted dose. Perhaps two injections provide enough NCM to decrease IL1β levels in these donors. The extent to which IL1β decreased, or in some cases increased, was not uniform throughout all donors. In some donors a drastic IL1β decrease could be seen, whereas others showed little change. This can possibly be assigned to donor variation.

The results showed that IL1β protein levels were generally higher in NX IVDs in comparison to no NX IVDs. NX is a way to induce IVD inflammation and degeneration and therefore higher protein levels of IL1β in NX IVDs were expected. When comparing treatment of NX and no-NX IVDs, no-NX IVDs generally showed a slight decrease of IL1β in some donors. In three out of the five donors, NX IVDs showed higher IL1β protein levels with each injection. A possible hypothesis could be that NCM is able to control mild inflammation with relatively lower IL1β protein levels, such as in the no-NX IVDs, whereas it is not potent enough to exert an effect on the moderately degenerated IVD in which the IL1β protein levels are higher. However, it is important to emphasize that there was a lot of donor variation and the decrease in the IL1β protein levels in the no-NX IVDs was only slight and not significant. Hence, no definitive conclusions can be drawn from these results.

An interesting finding in the human field is that lower levels of IL1β can actually induce a reparative response (Philips et al, 2015). Research of Philips *et al.* (2015) found increased aggrecan levels corresponding with lower IL1β protein levels, ranging from 0,001 to 0,1 ng/mL. Interestingly, once the concentration of IL1β was 1 ng/mL or higher, aggrecan levels did decrease (Philips et al, 2015). Based upon these results, the increased IL1β levels by NCM injections, as found in this part of the study, do not necessarily mean that they have a negative impact on the IVD. Lower protein levels of IL1β could actually have positive effects as found in human IVDs. However, it must be stipulated that these suggestions are hypothetical and therefore more research on the subject is necessary.

Limitations and future studies

One of the limitations of this study was that all donors received the same treatment in the same IVD segment. The effect of NCM can differ between segments and stages of degeneration. Therefore it could be interesting to look at the effect of NCM in different IVD locations in the spinal cord, as each segment can possibly react differently. Another limitation was the donor variation. Only five donors were available for this study, which showed quite some variation in their response. The number of donors would ideally be expanded to a bigger group. This would be useful information, for when this therapy reaches application in the clinic. Once this will be applied as a form of therapy, it is good to know that results might vary in the different canine patients. This makes research on this subject valuable. Besides, it must be emphasized that microscopic images and cell counts were performed manually. This could have an effect on the results up to a certain level, even though fields were randomly chosen for imaging and cell counts. Besides, cell counts were performed in the same fashion as mentioned in part two of this thesis. Therefore, only a limited part of the NP was covered and additional microscopic images are needed to result in a more representative positive cell ratio.

Future research could focus on the effect NCM has on other cytokines, such as IL8 expression or TNFα protein levels. As already stated before, IL1β enhances IL8 gene expression (de Vries et al, 2019), which is also associated with the inflammation process and IVD degeneration. Studying other cytokines could provide more insight into the effect of NCM on IVD degeneration.

Clinical research could look into the effect the process of injecting itself has. This could be done by, for example, injecting a placebo such as physiological saline solution to see if this triggers an inflammatory reaction. This can be done in for example, CD donors, healthy donors and donors with induced IVD degeneration through nucleotomy, to provide the most precise results.

Options for future research, beside the histological evaluation of IL1β, are performing an ELISA, qPCR or intracellular inflammatory cytokine analysis with flow cytometry. This can detect antibodies, DNA or replication status of cells respectively. Adding these read-out parameters will result in a more complete data set, which will provide the opportunity to draw more concrete conclusions.

Advancement in this research field is important as it can be translated to human medicine in a later stage. Once working mechanisms of NCM can be elucidated in canines, the translation to humans could provide a great breakthrough of solving lower back pain problems in both humans and our canine companions.

Conclusion

In this part of the study the effect of NCM on the IL1β protein levels in degenerated canine IVDs was analysed. Although not significant, the NPs of the moderately degenerated IVDs showed higher IL1β protein levels in comparison to mild degeneration. This is in line with the expectation that NX induces inflammation. Despite the donor variation, NPs of the IVDs with moderate degeneration generally showed an increase of IL1β protein levels with each NCM injection. In contrast, some NPs of the no-NX IVDs showed a slight decrease in IL1β protein levels. These results suggest that NCM is able to control mild inflammation with relatively lower IL1β protein levels, whereas it is not potent enough to exert an effect on the moderately degenerated IVDs in which IL1β protein levels are higher. However, the question remains whether the IL1β levels should be considered to stimulate a catabolic or potentially an anabolic process. Therefore, it would be interesting to focus on more and different inflammatory cytokines in future research, to gain more insight on the anti-inflammatory effect of NCM. Once working mechanisms of NCM can be elucidated in canines, the translation to human patients could provide a great breakthrough of solving lower back pain problems in both humans and our canine companions.

General conclusion

The aim of this study was to analyse IL1β protein levels in canine intervertebral disc degeneration. IL1β were first analysed in NCD puppy and adult dogs and in CD dogs NPs. Results showed that IL1β protein levels were the lowest in NCD puppies and the highest in CD dogs, which suggest that IL1β protein levels increase during IVD degeneration. This was in line with expectations and previous research. These results were also interesting for the last part of this study, where the effect of NCM injections on IL1β protein levels in the NPs degenerated beagle IVDs was analysed. NC-rich NPs of the NCD breeds appeared to have lower IL1β protein levels and the donors in the last part of the study received NC-rich NP matrix injections. Therefore it was interesting to see if NCM injections lowered IL1β protein levels in the NP, as a possible therapy of IVD degeneration. As expected, the NPs of moderately degenerated IVDs did show higher IL1β protein levels compared with mild degeneration. However, this was not significant. These results do suggest a correlation between IL1β protein levels and the degree of degeneration in the IVD. Surprisingly, NPs of the IVDs with moderate degeneration showed increased IL1β protein levels with each NCM injection. Although, this might be a positive result as IL1β can possibly cause a reparative response. Future research is needed, as donor variation and a limited donor group prohibited the formation of definitive conclusions. In addition to this, studying other cytokines involved in the degenerative process of the IVD, such as IL6 and IL8, can provide further insight into the role of inflammation and the effects of NCM. Further elucidating this subject can provide necessary results for the translation to human medicine and developing a successful treatment options for both humans and canines.

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