



CANINE INTERVERTEBRAL DISC DEGENERATION AND NUCLEUS PULPOSUS CELL CULTIVATION

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AUTHOR	SUPERVISOR
C.L.E. (Caroline) Hoogeveen	Dr. F.C. (Frances) Bach
5652804	Department Clinical Sciences
<u>c.l.e.hoogeveen@students.uu.nl</u>	Companion Animals
	Faculty of Veterinary Medicine
	Utrecht University, The Netherlands

GENERAL INTRODUCTION

LOW BACK PAIN

Low back pain is a common problem amongst humans and dogs and is associated with socioeconomic consequences such as discomfort and healthcare costs.^{1,2} Intervertebral disc (IVD) degeneration is a frequent cause of chronic low back pain.^{3,4} In dogs, neurological problems can be caused by IVD degeneration. It can lead to various diseases, such as disc herniaton, degenerative lumbosacral stenosis and cervical spondylomyelopathy.^{4,5} Diseases related to IVD degeneration cause a large part of euthanasia in dogs under 10 years old.¹

THE HEALTHY INTERVERTEBRAL DISC

In both humans and dogs, the IVD is a fibro-cartilaginous structure between the vertebral bodies. Its function is to withstand and transmit physiological spinal pressure that is put on the vertebrae while allowing mobility.⁶ The IVD is composed of three distinct regions, the inner gel-like nucleus pulposus (NP), the outer annulus fibrosus (AF) and the cartilaginous endplates (CEPs) that connect the IVD to the adjacent vertebrae. The region in which the NP passes into the AF or CEPs is called the transition zone (TZ).⁷

In the juvenile healthy NP, the most common cell is the notochordal cell (NC), which is characterized by large cytoplasmic vesicles.^{4,8} These cells are found in clusters and produce the extracellular matrix (ECM) of the NP, which is rich in proteoglycans and collagen type II.^{4,8} The proteoglycans consist of a protein backbone with negatively charged glycosaminoglycan (GAG) side chains and are aggregated with hyaluronic acid, resulting a strong osmotic gradient, which attracts a large amount of water into the NP.⁴ The inner layers of the AF consist of fibrocytes and chondrocyte-like cells, with the ECM containing mostly collagen type II. The outer layers contain fibrocyte-like cells and mainly collagen type I.^{4,8} The CEPs contain chondrocyte-like cells and its biochemical composition is very similar to that of articular cartilage.⁴

INTERVERTEBRAL DISC DEGENERATION

IVD degeneration is a deviating, cell-mediated response to progressive structural failure. It is associated with chronic loading/abnormal biochemical stresses, trauma and genetic predisposition.⁴ In dogs, IVD degeneration is characterized by replacement of large vacuolated NCs by smaller non-vacuolated clustered chondrocyte-like NP cells (NPCs), a process referred to as chondrification.⁴ Due to this cell shift, a change in ECM components is seen.⁹ Loss of NCs results in a decrease in proteoglycan content and a degradation of GAG molecules.⁴ NPCs and their associated ECM resemble hyaline cartilage, which consists mainly of disorganized collagen fibers.⁴ As a consequence of this shift, the strength of the IVD reduces and the IVD is no longer capable of withstanding physiological pressures properly.⁹ This is seen by cleft and cracks, a decreased height and even bulging or herniation of the IVD.⁴

In humans, major age-related changes in the IVD, such as cell type changes, cleft and tears, are reported to occur at an age of 10.¹⁰ Around 6-10 years, loss of almost all NCs has been seen.¹¹ The changes due to the pathological process of degeneration are similar to age-related changes, making it difficult to differentiate IVD degeneration from the phenomenon of aging in humans.¹⁰ For this reason, in humans, the changes that occur within the IVD are referred to as maturation instead of degeneration.

Similar changes have also been reported in dogs at an age of 12-60 months, depending on the breed. Dogs can be divided into two groups based on predisposition to chondrodystrophy, as was first described by Hansen in 1952.⁸ In chondrodystrophic (CD) dog breeds, such as the Dachshund and Beagle, endochondral ossification of primarily the long bones is disturbed, resulting in disproportionally short extremities.¹² IVD degeneration is more common in these breeds. The degenerative processes in CD dog breeds occur at a higher rate than in non-chondrodystrophic (NCD) dog breeds.¹³ In CD dogs, IVD degeneration typically occurs at an age of 3-7 years. In NCD dogs, the disease develops around 6-8 years.¹² In general, more NPCs and less NCs are seen in the NPs of CD breeds. However, replacement of NCs by NPCs is seen in both groups.¹³

CANINE ANIMAL MODEL

Since IVD-induced back pain is such a common problem, there is great need for an animal model that represents the human conditions. A study done by Bergknut et al. (2012) investigated the suitability of CD and NCD dogs to serve as a model for human IVD degeneration by comparing the morphological appearance, histological structure, and biochemical characteristics in different stages of IVD degeneration in dogs and humans.¹ They found that the gross pathology and histopathology of canine IVDs and human IVDs were similar in all different stages of IVD degeneration.¹ Therefore, both CD and NCD dog breeds are considered a good animal model for human IVD degeneration.

Various scales have been developed in order to classify the degree of IVD degeneration. Macroscopically, pathological changes during degeneration of the IVD can be divided into five categories, as described by Thompson et al. (1990).¹⁴ This grading scheme describes five grades, with grade 1 being the least degenerated stage and grade 5 the most, considering the morphology of the NP, AF, CEPs and adjacent vertebral bodies. Even though this grading scheme was developed for evaluation of the human IVD, it has also been found that it is suitable to be used in canine IVD degeneration.¹⁵ In 2001, an MRI grading system for the evaluation of IVD degeneration was developed by Pfirrmann et al. (2001).¹⁶ It also ranges from grade 1 to grade 5, with grade 5 being the most degenerated stage. The system considers structure and signal intensity of the NP, distinction clarity of the NP and AF and the height of the IVD.¹⁶ Microscopically, Bergknut et al. (2013) developed a histological grading scheme (modified from the human Boos grading scheme¹⁷) for canine IVD degeneration based on nine different histological variables, being the morphology of AF, chondrocyte metaplasia in AF, tears and cleft formation, chondrocyte proliferation of the NP, presence of NCs in the NP, matrix staining of the NP with Alcain blue/Picrosirius red staining, endplate morphology, new bone formation and subchondral bone sclerosis.¹⁸

CURRENT TREATMENT STRATEGIES

Current treatment strategies in both humans and dogs mainly focus on alleviating pain by medication and/or physiotherapy. Since degenerated NP tissue produces inflammatory mediators, anti-inflammatory drugs are indicated as well.¹⁹ Furthermore, surgical techniques could be used to release pressure on neural structures, in case conservative treatment does not have the desired effect (anymore).²⁰ These current therapeutic options are palliative and not curative, resulting in many patients continuing to have symptoms. In the last few years, regenerative treatment strategies, aimed at the biological repair of the degenerated IVD, have been investigated and seem promising.²⁰ The regenerative potential of the NC and its matrix is increasingly being researched and substantiated.²¹ In addition, it has also been demonstrated that NPCs can be used to suppress IVD degeneration.²² Since the NP is the most affected region of the IVD during degeneration²³, research into new treatment methods is often focused on the repair of this area.

THE ROLE AND EXPRESSION OF PAX1 IN CANINE NUCLEUS PULPOSUS CELLS

In order to use cells of the NP for regenerative purposes, understanding of the normal NPC phenotype is necessary.²⁴ This is important since knowledge of phenotypic characteristics, rather than merely a genotype, helps to understand physiologic processes and NP cell function.²⁵ Several studies have attempted to define mainly the human NPC phenotype by identifying "markers"-genes, proteins and metabolic characteristics, including Paired box 1 (PAX1).^{23,24,26,27} The PAX1 gene encodes a transcription factor PAX1 protein that regulates the development of vertebral structures during embryogenesis.²³ PAX1 protein is thus a transcriptional activator and is expressed in the nucleus of the cell. A study done by Wallin et al. (1994) showed that mice with a point mutation in the PAX1 gene developed an anomalous vertebral column with reduced or even absent intervertebral discs.²⁸

AIM

This paper consists of two parts. The aim of the first part is to determine PAX1 expression in different stages of canine IVD degeneration by looking at immunopositivity. The aim of the second part is to investigate the suitability of different cell culture media combinations for cultivation of canine NPCs by looking at PAX1 immunopositivity of various cultured canine NPCs.

PART 1. PAX1 IMMUNOPOSITIVITY IN CANINE INTERVERTEBRAL DISC DEGENERATION

ABSTRACT

Background – Little is known about phenotype markers of the canine nucleus pulposus cell (NPC). Because of remarkable similarities regarding intervertebral disc (IVD) degeneration between dogs and humans, it is likely that the phenotype is alike as well. Paired box 1 (PAX1) is a marker for the healthy human NPC, whose expression decreases as the degree of degeneration increases. Nothing is yet known about the expression of PAX1 during canine IVD degeneration.

Objective – To investigate PAX1 expression in different stages of IVD degeneration in dogs.

Methods – Three decalcified canine IVD samples per Thompson grade were used, from both chondrodystrophic and non-chondrodystrophic dog breeds. Immunohistochemical staining was performed and PAX1 immunopositivity ratio was determined for every sample.

Results – An increasing pattern in PAX1 immunopositivity from grade 1 towards grade 5 was visible. No significant differences in PAX1 expression were found between different Thompson grades. A significantly strong positive correlation between PAX1 immunopositivity ratio and IVD degeneration grade was found, indicating that the higher the Thompson score, the higher the PAX1 expression in canine NPCs.

Conclusion – The correlation that was found in this study does not correspond to the human pattern. In fact, it is the opposite. This could either indicate that PAX1 is not a healthy canine NPC marker or that there is a reason for PAX1 expression to increase, such as a reparative response of the canine NPCs. Furthermore, low sample size could have affected the outcome of this study. Hence, more future research into the role of PAX1 and its expression in canine NPCs is required.

INTRODUCTION & AIM

Unlike the human NPC phenotype, which is increasingly being studied, little is known about markers for healthy canine cells of the NP: NCs and NPCs. Since there are remarkable similarities between human and canine IVD degeneration¹, it is plausible that the human NPC phenotype is comparable to the canine one. However, as previously mentioned, only NPCs are seen in the adult human IVD, while NCs are still present in the adult canine IVD as well (mainly in grade 1 and a little in grade 2 according to the Thompson scale).¹¹

As previously mentioned, PAX1 has been described as a marker for the healthy human NPC.^{23,24,26,27} The expression of PAX1 in the healthy human IVD and in IVD degeneration has been investigated by various studies. They showed that PAX1 mRNA and PAX1 protein is 1.2-1.5–fold higher in cultured NPCs compared to AF cultures.^{26,27} Regardless of degeneration grade, PAX1 mRNA and PAX1 protein expression is significantly higher in NPCs in comparison to articular chondrocytes (ACs), where the gene expression level for PAX1 is >1,000-fold higher in NPCs than in ACs.²³ No significant regional variation (cervical vs lumbar discs) regarding PAX1 expression has been identified.²⁷ A significantly lower gene expression for PAX1 in mature adult in comparison to young adult specimens has been demonstrated.²⁷ Furthermore, despite the fact that PAX1 is present in all stages of degeneration, PAX1 expression shows a significant decrease from moderately to severely degenerated human NP.²⁷

The aim of this part of the study was to investigate PAX1 expression in canine IVDs of different degeneration grades. This was determined by applying PAX1 immunohistochemistry on decalcified canine IVD samples. The expression of PAX1 in canine IVD degeneration is expected to be comparable to humans, because of the mentioned similarities in terms of IVD degeneration.¹ Since the human NP contains only NPCs (regardless of degeneration grade)¹¹, PAX1 expression in NCs has not been determined. PAX1 has been indicated as a healthy NPC marker, leading to the hypothesis that PAX1 would also be present in NCs.

MATERIALS & METHODS

STUDY POPULATION AND SAMPLE PREPARATION

Three decalcified canine IVD samples per Thompson grade were used. The IVDs were derived from both CD dog breeds and NCD dog breeds of different ages (Table 1). These samples are a part of the samples as used in Bergknut et al. (2012)¹⁸. All dogs were research dogs that had been euthanized in unrelated studies or were client-owned dogs that were submitted to the Department of Pathobiology at the Faculty of Veterinary Medicine, Utrecht University. Permission to use the spine was granted by the owners. None of the dogs had a reported history of back problems.

After dissection, the spines were transected in the midsagittal plane. High-resolution photographs of each spinal unit (endplate-intervertebral disc-endplate) were used for grading according to the Thompson scale¹⁴. Midsagittal slices (3-4 mm thick) were cut and the segments were fixed in 4% neutral buffered formaldehyde solution and subsequently decalcified in ethylenediaminetetraacetic acid (EDTA). After decalcification, all samples were embedded in paraffin.

Sample number	Thompson grade	Breed (NC/C)	Age (months)
1	1	NC	17
7	1	NC	16
23	1	NC	36
3	2	С	25
5	2	С	25
11	2	NC	84
14	3	NC	120
15	3	NC	120
26	3	С	117
13	4	NC	84
30	4	С	120
32	4	С	120
20	5	С	192
33	5	С	120
34	5	С	120

 Table 1 – Overview samples. NC = non-chondrodystrophic, C = chondrodystrophic.

IMMUNOHISTOCHEMISTRY

The protocol that was used for immunostainings was based on the method by Binch et al. (2020)²⁹. This means that two types of antibodies were used; the primary antibody that binds to the target antigen and the horseradish peroxidase (HRP)-bound secondary antibody that binds to the primary antibody. After application of the substrate 3,3'-diaminobenzidine (DAB), HRP converts DAB, resulting in a dark brown precipitate. Hence, brown discoloration indicates the presence of the target antigen, which in this case is the PAX1 protein. The staining protocol for PAX1 has been optimized by running several tests with small adjustments each time. The elaboration of the optimization and details of the protocol can be found in Supplementary file 1.

After deparaffinizing, the slides were washed in phosphate-buffered saline (PBS) for 5 minutes. Endogenous peroxidases were blocked by applying Dako Dual Endogenous Enzyme Block on the slides for 10 minutes at room temperature. The slides were then washed two times with PBS 0.1% Tween (PBS-T). Heat-mediated antigen retrieval was performed for 30 minutes using a 10 mM citrate buffer (pH 6) in a 70°C water bath. After cooling down, nonspecific protein interactions were blocked by 5% PBS/BSA (Bovine Serum Albumin), which was left on the slides for 30 minutes. After this, the slides were incubated with primary PAX1 rabbit polyclonal antibodies (Abcam, ab203065, 1 mg/mL) with a concentration of 20 μ g/mL (dilutions performed in 5% PBS/BSA) and left overnight at a temperature of 4°C.

The next day, the slides were washed two times with PBS-T 0.1% and incubated with rabbit secondary antibodies conjugated with HRP for one hour. Thereafter, the slides were washed again two times with PBS and incubated in Bright DAB substrate kit until brown discoloration was seen. The slides were then briefly rinsed in demi water to stop the DAB reaction. Next, counterstaining was done with Hematoxylin QS for 10 seconds, followed by a rinse in tap water for 10 minutes. Finally, the slides were dehydrated and covered. Positive control slides (non-decalcified canine NP tissue) were included and showed specific staining.

MICROSCOPIC AND STATISTICAL ANALYSIS

10x and 20x magnified microscopic images were taken by the use of The Olympus BX43 light microscope. Of each sample, two random 20x magnified images of the NP were used for evaluating PAX1 immunopositivity ratio (%positive cells). IBM SPSS Statistics 22 was used for statistical analyses. The data were examined for normal distribution through the Shapiro Wilks test. Since the data were not normally distributed, Kruskal-Wallis and Mann-Whitney U tests were performed to determine differences in PAX1 immunopositivity per Thompson grade. For the correlation between PAX1 immunopositivity ratio and IVD degeneration grade, the Spearman's rank test was used. P-values <0.05 were considered significant.

RESULTS

Brown discoloration, indicating PAX1 expression, was detected in every Thompson grade, but there appeared to be considerably more PAX1 immunopositivity in higher grades of degeneration (Thompson grade 4 and 5) (Fig. 1.1). There was a trend visible in terms of the degree of positive PAX1 staining; increasing from grade 1 towards grade 5.

When comparing PAX1 immunopositivity ratio between different Thompson grades, there were no significant differences in PAX1 expression (p=0.100) (Fig. 1.2). This is presumably due to low sample size (n=3 for each Thompson grade). However, a positive trend was seen.

There was a significant positive correlation between PAX1 immunopositivity ratio and IVD degeneration grade (r=0.814; p=0.0002) (Fig. 1.3). This indicates that the higher the Thompson grade, the higher the PAX1 expression in canine NPCs (strong correlation).



Fig. 1.1 – Microscopic images of canine nucleus pulposus cells immunohistochemically stained for PAX1 (n=15). Grades according to Thompson scale. White arrows indicate examples of negative cells, black arrows indicate examples of positive cells. Bars indicate 50 μm.



Fig. 1.2 – Mean and standard deviation of canine nucleus pulposus cell PAX1 immunopositivity ratio per Thompson grade (n=15).



Fig. 1.3 – Canine nucleus pulposus cell PAX1 immunopositivity ratio plotted against Thompson grade per sample (n=15).

CONCLUSION & DISCUSSION

The aim of this study was to investigate PAX1 expression in different stages of canine IVD degeneration. PAX1 expression has been studied in mostly human NPCs. Research shows that PAX1 is expressed in NPCs at all stages of human IVD degeneration.²⁴ However, PAX1 expression shows a significant decrease in human NPCs as degeneration increases, which indicates that PAX1 is a healthy NPC marker.²⁷ In addition, in humans, NPCs of mature IVDs show a lower PAX1 gene expression than those of juvenile IVDs.²⁷ The results of this study do not correspond to the reported human pattern. In fact, in this study, it seems that PAX1 expression increases in canine NPCs, as degeneration and age progress. This is an interesting outcome, since the opposite was expected due to IVD degeneration similarities between dogs and humans. In the human study²⁷, a different grading scheme was used than the one that was used in this study. This could slightly affect the comparison, however, it is not expected that it would produce such differences.

The fact that PAX1 expression seems to be lower in grade 1, 2 and 3 than in grade 4 and 5 raises the question whether PAX1 is indeed a healthy canine NPC marker or that the expression pattern of PAX1 differs between humans and dogs. Furthermore, it appears that PAX1 is not an NC marker, since no PAX1 immunopositivity was found in these cells. Whether PAX1 is a marker of NPCs could be investigated by comparing their PAX1 expression to the PAX1 expression in, for example, cells in the AF or in ACs. It might be that PAX1 is a canine NPC marker, however, instead of a healthy marker, it could be that it is a degeneration marker, since the expression increases during degeneration. In order to determine healthy canine markers, researchers in future studies should look into the canine NC and NPC phenotype by identifying healthy cell markers, as was done for human cells.^{23,24,26,27}

Assuming PAX1 is a healthy canine NPC marker (as it is in humans), the increase in PAX1 immunopositivity in degenerated IVD samples could indicate a response of the cells to the damaged surrounding tissue. This implies that the increase of PAX1 is a result of an anabolic reparative response of the NPCs, as also observed for caveolin-1 immunopositivity³⁰. Brown et al. (2018) noted that small cell clusters in the AF are indicative of stem cell activity and a reparative response.³¹ Since PAX1 is expressed in cells in the AF as well^{26,27}, researchers could look at whether PAX1 expression occurs particularly in these cell clusters during degeneration, so as to provide more scientific support for the reparative response hypothesis.

To be able to shed some light on the role of PAX1, it would be interesting to look at other proposed NPC markers, such as Forkhead box F1 (FOXF1)²⁴, and compare their expression pattern during canine IVD degeneration with PAX1. When expression of multiple healthy markers increases with degeneration, a reparative response would be more likely.

The PAX1 immunopositivity ratio was determined by counting the (positive) cells manually. With immunohistochemistry, evaluating whether or not a cell is positive is subjective and could vary between different investigators. Furthermore, in this study, three samples per Thompson grade were used. This is a relatively small sample size, which might affect statistical outcome. Therefore, it might be interesting for researchers to investigate PAX1 expression in canine IVD degeneration in a larger sample size in the future, based on power analysis. These limitations should be taken into account when interpreting the results of this study.

PART 2. PAX1 IMMUNOPOSITIVITY IN CULTURED CANINE NUCLEUS PULPOSUS CELLS

ABSTRACT

Background – For canine nucleus pulposus cell (NPC) cultivation, expansion and redifferentiation media containing fetal bovine serum (FBS) are commonly used. However, due to several disadvantages of FBS, possibilities for reduction of FBS or alternatives are increasingly being sought. Fibroblast growth factor (FGF) might be such an alternative. Cultured cells should express healthy NPC markers. In human cells, Paired box 1 (PAX1) is such a healthy marker. Due to similarities between humans and dogs regarding intervertebral disc degeneration, PAX1 expression was evaluated in canine NPCs cultured in different conditions.

Objective – To investigate which expansion medium and redifferentiation medium could be best combined in order to grow the healthiest canine NPCs, considering the amount of FBS during expansion and redifferentiation and the presence of FGF during expansion.

Methods – Canine NPCs were collected from three Beagles and cultured in six different conditions; three different expansion media (10% FBS, 5% FBS + FGF and 5% FBS) and two different redifferentiation media (10% FBS and no FBS). Immunohistochemical staining was performed and PAX1 immunopositivity was evaluated for every condition.

Results –PAX1 immunopositivity did not vary between different conditions in terms of expansion media. In terms of redifferentiation, cells cultured in media with FBS showed slightly more positive PAX1 staining. FGF did not have an effect on PAX1 immunopositivity or the amount of cells.

Conclusion – Considering the aim of reducing the use of FBS, it would be best to use an expansion medium containing 5% FBS, since fewer FBS does not affect PAX1 expression. Regarding redifferentiation, a medium with 10% FBS could best be used for culturing canine NPCs. However, since it is uncertain whether PAX1 is a healthy canine NPC marker, other readouts should be considered as well.

INTRODUCTION & AIM

Since dogs can serve as an animal model for human IVD degeneration research¹, canine tissue can be used to investigate therapeutic options for both humans and dogs. Research into treatment options is initially done *in vitro*. Developed methods are then extrapolated to *in vivo* animal models before being used in the clinic.²⁰ Intradiscal injections of allogenic NPCs appear to inhibit IVD degeneration.²² In addition, there are clinical trials using such cell-based therapies.³² These treatment strategies require enough cells, while the degenerated tissue from which cells are extracted contains few cells. For this reason, expansion of NPCs is necessary for both *in vitro* and *in vivo* therapy research.

Cultivation of NPCs can be done in different expansion media (to gain enough cells for experiments) combined with different redifferentiation media (to regain a healthier NPC phenotype after expansion), since expansion has been shown to dedifferentiate NPCs. Cell-containing alginate beads is a commonly used 3D cell culture model. This model can be used for suspension of cells of the NP (either NCs or NPCs) from canine spines. Generally, NPCs are expanded and redifferentiated in 10% fetal bovine serum (FBS). FBS is commonly used in cell culture applications. However, the use of FBS has a number of drawbacks. It is obtained from a living calf fetus, which poses problems regarding animal welfare.³³ Furthermore, several disadvantages of FBS regarding quality and reproducibility of data are reported.³³ These features make it more desirable to grow cells with less or no FBS.

The effect of fibroblast growth factor (FGF) during cell cultivation has been investigated on different types of (human) cells and it has been shown that FGF amplifies the cell proliferation and expansion.³⁴⁻³⁶ FGF might therefore be suitable to replace part of the FBS during expansion.

The aim of this part of the study was to investigate which expansion medium and redifferentiation medium could be best combined in order to grow the healthiest canine NPCs. Because PAX1 is expected to be a healthy NPC marker^{23,24,26,27}, cells should therefore express this protein abundantly when cultured in favorable conditions. Different amounts of FBS during expansion and redifferentiation and whether or not to use

FGF during expansion were considered. The addition of FGF might lead to more cells, since FGF enhances cell expansion.³⁴⁻³⁶ However, since FGF is a growth factor for fibroblasts, it might be that the NPCs become more fibroblast-like and thus show less PAX1 expression. In order to determine which expansion and redifferentiation conditions result in the healthiest NPC phenotype, PAX1 immunopositivity was determined in various canine NPCs, cultured in six different culture conditions (three different expansion media and two different redifferentiation media).

MATERIALS & METHODS

STUDY POPULATION AND SAMPLE PREPARATION

For this study, complete spines were collected from three female Beagles (B1, B2 and B3), 3-4 years of age, that had been euthanized in unrelated research studies (approved by the Utrecht University Animal Ethics Committee, DEC study number 2016.II.529.002). The Beagle is a CD breed and these dogs develop IVD degeneration at about one year of age and therefore are considered a good model for human research.¹ At an age of 3-4 years, Beagles have almost only chondrocyte-like NPCs and a negligible number of NCs.¹² NP tissue was collected and underwent digestion and NPCs were obtained as previously described³⁷.

First, NPCs were expanded. Three different expansion media were tested for cell culture expansion:

- 1. Expansion medium 1 **10% FBS**: hgDMEM+Glutamax with 10% FBS, 0.1 nM Ascorbic acid 2-phosphate (Asap; A8960, Sigma-Aldrich) and 0.5% Fungizone (15290-018, Invitrogen).
- Expansion medium 2 5% FBS + FGF: hgDMEM+Glutamax with 5% FBS, 1 ng/mL bFGF (PHP105, AbD Serotec), 0.1 mM Asap, 0.5% Fungizone, 1% ITS+ premix (354352, Corning Life Sciences), 0.04 mg/mL L-proline (P5607, Sigma-Aldrich), and 1.25 mg/mL Human Serum Albumin (HSA, Sanquin Research).
- 3. Expansion medium 3 **5% FBS**: hgDMEM+Glutamax with 5% FBS, 0.1 mM Asap, 0.5% Fungizone, 1% ITS+ premix, 0.04 mg/mL L-proline and 1.25 mg/mL HSA.

All cells were expanded at approximately 5000 cells/cm² at 5% O₂, 5% CO₂, 37 °C. Expansion medium was changed twice weekly.

After the canine NPCs (expanded in all three conditions) reached 80-90% confluence in P2, they were suspended in filter-sterilized 1.2% alginate (180947, Sigma-Aldrich) beads of approximately 15-20 μ L at 4*10⁶ cells/mL. Two different redifferentiation media were tested:

- 1. Redifferentiation medium 1 **FBS**: hgDMEM+Glutamax with 10% FBS, 0.1 mM Asap, 0.5% Fungizone.
- 2. Redifferentiation medium 2 **no FBS**: hgDMEM+Glutamax with 0.1 mM Asap, 0.5% Fungizone, 1% ITS+ premix, 0.04 mg/mL L-proline, and 1.25 mg/mL HSA.

NPC-containing beads were cultured for 14 days at 5% O₂, 5% CO₂, 37°C.

In total, six different conditions per donor were tested; three different expansion media and two different redifferentiation media (Table 2). Six beads were cultured per well in a 24-wells plate (CLS3473-24EA, Corning) in 700 μ L redifferentiation medium. After 0 and 14 days, three beads (n=2 replicates per donor and condition) were pooled for histological purposes.

Culture condition number	Expansion medium	Redifferentiation medium
1	10% FBS	FBS
2	10% FBS	No FBS
3	5% FBS + FGF	FBS
4	5% FBS + FGF	No FBS
5	5% FBS	FBS
6	5% FBS	No FBS

Table 2 – Overview culture conditions for canine nucleus pulposus cells, FBS = fetal bovine serum, FGF = fibroblast growth factor. N=3 Beagles per condition.

IMMUNOHISTOCHEMISTRY AND MICROSCOPIC IMAGING

For immunostainings, the same protocol that was used in part 1 of this paper was now applied to these cells. This time a concentration of 4 μ g/mL polyclonal rabbit antibody (Abcam, ab203065, 1 mg/mL) was used. 20x magnified microscopic images were taken by the use of The Olympus BX43 light microscope in order to evaluate PAX1 immunopositivity. This was assessed by looking globally at which cells showed the most brown discoloration.

RESULTS

When cells cultured in different expansion media were compared at day 0 (Fig. 2.1), it seemed that there was no optimal expansion medium for canine NPCs, considering the amount of expression of PAX1. Unfortunately, all cells of donor B2 at day 0 were lost during the staining process. As for day 14 (Fig. 2.2-2.4), after redifferentiation, there was no clear difference seen in PAX1 immunopositivity between cells grown in different expansion media as well. Regarding cells derived from donor B1 (Fig. 2.2), there was slightly more positive staining present in the culture conditions containing 10% FBS (culture condition 1 and 2). However, this was not seen in the cells of donor B2 and B3.

The influence of FGF could be determined by comparing the results of 5% FBS with FGF (culture condition 3 and 4) and 5% FBS without FGF (culture condition 5 and 6). When looking at those culture conditions, no obvious difference was seen regarding PAX1 immunopositivity and the amount of cells. However, unfortunately, the cells that underwent culture condition 5 were lost of two donors, making it harder to compare.

When comparing different redifferentiation media, slightly more positive staining was present in the cells that were cultured in redifferentiation media with FBS (culture condition 1, 3 and 5) regarding the cells cultured in redifferentiation media without FBS. This trend was generally visible.

Day 0 – Donor B1 and B3



Fig. 2.1 – Day 0 donor B1 and B3. Microscopic images of cultured canine nucleus pulposus cells immunohistochemically stained for PAX1. E = expansion medium, FBS = fetal bovine serum, FGF = fibroblast growth factor. Bars indicate 50 μm.



Day 14 – Donor B1

Fig. 2.2 – Day 14 donor B1. Microscopic images of cultured canine nucleus pulposus cells immunohistochemically stained for PAX1. E = expansion medium, R = redifferentiation medium, FBS = fetal bovine serum, FGF = fibroblast growth factor. Numbers in images indicate culture conditions. Bars indicate 50 μ m.





Fig. 2.3 – Day 14 donor B2. Microscopic images of cultured canine nucleus pulposus cells immunohistochemically stained for PAX1. E = expansion medium, R = redifferentiation medium, FBS = fetal bovine serum, FGF = fibroblast growth factor. Numbers in images indicate culture conditions. Cells of culture condition 5 were lost during the staining process. Bars indicate 50 μ m.



Day 14 – Donor B3

Fig. 2.4 – Day 14 donor B3. Microscopic images of cultured canine nucleus pulposus cells immunohistochemically stained for PAX1. E = expansion medium, R = redifferentiation medium, FBS = fetal bovine serum, FGF = fibroblast growth factor. Numbers in images indicate culture conditions. Cells of culture condition 1 and 5 were lost during the staining process. Bars indicate 50 μ m.

CONCLUSION & DISCUSSION

The aim of this study was to determine the most favorable combination of media to expand and redifferentiate canine NPCs, based on PAX1 immunopositivity. Based on these study results, there is not enough evidence to conclude that there is an optimal culture condition in terms of expansion media. PAX1 immunopositivity of some images vary, but do not remarkably differ; no clear trend was seen in all donors in PAX1 immunopositivity of cells regarding different expansion media.

It has been shown that FGF causes enhanced proliferation and expansion in human cells.³⁴⁻³⁶ However, the results of this study do not show a remarkable difference between canine cells cultured in expansion media with and without FGF, regarding both PAX1 immunopositivity and the amount of cells. Based on the results of this study, there is no reason to assume that adding FGF to the expansion medium either has a positive or negative effect on canine NPC cultivation. It might be a suitable alternative for FBS, since there was no clear difference in PAX1 immunopositivity between the culture conditions with 10% FBS and 5% FBS with FGF. For this reason, it would be interesting to include an expansion medium containing only FGF in the future.

As mentioned before, FBS is a widely used supplement for cell cultivation. Because of several disadvantages, such as ethical concerns and contamination issues, the search for alternatives has gained increased attention.³³ However, no proper serum-free alternatives have been developed. Therefore, it might be a good first step to use less FBS, until serum-free alternatives are developed. The results of this study show that less FBS (5% instead of 10%) does not affect the PAX1 immunopositivity of cultured canine NPCs.

In terms of redifferentiation media, there was a difference in PAX1 immunopositivity visible between the different conditions. Overall, cells cultured in redifferentiation media with FBS showed more positive PAX1 staining than those cultured in redifferentiation media without FBS. This indicates that redifferentiation media containing FBS are preferred over redifferentiation media without FBS when used for canine NPC cultivation. However, it should be kept in mind that some cells were lost.

Since it does not seem to matter whether 10% FBS or 5% FBS is used for the expansion medium, it might not matter either for the redifferentiation medium. In this study, 10% FBS was used for redifferentiation. However, more research could be done on the minimum percentage FBS needed for redifferentiation.

It can be concluded, based on the results, that the best combination is the expansion medium with 5% FBS with the redifferentiation medium with 10% FBS. As for the expansion medium, in this way, as little FBS is used as possible, as is preferred. As for the redifferentiation medium, this is because cells redifferentiated with FBS showed more positive PAX1 immunostaining.

This part of the study has its limitations as well, which should be kept in mind when interpreting the results. Based on part 1 of this study, it is not certain whether PAX1 is a healthy canine NPC marker. That also applies to this part of the study. In order to determine which culture conditions are best, other readouts, such as proliferation rate, other markers or ECM production, should certainly be considered as well. Furthermore, as mentioned before, some of the cultured cells were lost during the staining process. This includes one condition of donor B2 and two conditions of donor B3. This might affect the interpretation of the results. It would be interesting for future researchers to investigate the optimal culture conditions for canine NPCs by using more donors, so that the loss of some cells would have less impact on the results.

FINAL CONCLUSIONS

As a summary, PAX1 expression shows an interesting pattern in canine NPCs during IVD degeneration. Based on immunohistochemical staining, it is found that the expression of PAX1 in canine NPCs increases with higher grades of degeneration. In human NPCs, on the contrary, a decrease in PAX1 expression is seen during degeneration. The increase in the degenerated canine IVD might indicate that PAX1 is not a healthy canine NPC marker or that the increase in expression is a result of a reparative response of the NPCs.

For canine NPC cultivation, FGF seems to be a potential alternative for FBS, since no difference in PAX1 immunopositivity was seen between cells expanded in media with only FBS and in media with less FBS with the addition of FGF. Furthermore, it is possible to use 5% FBS for expansion instead of 10% FBS, which is commonly used now. For redifferentiation, a medium with FBS seemed more suitable for canine NPCs than a medium without FBS.

In order to assess the quality of canine NPCs cultured for *in vitro* and *in vivo* experiments, it is necessary to be familiar with the healthy NPC phenotype. For this, other healthy canine NPC markers should be investigated in the future. In addition, more information about the role of PAX1 in the canine NPC should be gathered in order to gain knowledge about the physiologic processes and function of the canine NPC.

SUPPLEMENTARY FILE 1. OPTIMIZATION OF PAX1 IMMUNOHISTOCHEMICAL STAINING

The slides that were used for optimization of the immunohistochemical staining were embedded in paraffin. The first protocol that was tested will be elaborated. Thereafter, the different adjustments done to this protocol will be explained. For every protocol, results will be discussed. In the first protocol that was used, the samples contained adult canine mixed breed (NCD) and Beagle (CD) NP tissue. The first step was to deparaffinize the slides. This was done in xylene (2 times 5 minutes), followed by 100% ethanol (2 times 3 minutes), 96% ethanol (2 times 1 minute) and 70% ethanol (2 times 1 minute). After this, the slides were washed in PBS for 5 minutes. Then endogenous peroxidases were blocked by applying Dako Dual Endogenous Enzyme Block on the slides for 10 minutes at room temperature. The slides were then washed two times with PBS-T 0.1% for 5 minutes. Next, nonspecific protein interactions were blocked by 5% PBS/BSA, which was left on the slides for 30 minutes. After this, the slides were incubated with primary PAX1 rabbit polyclonal antibodies (Abcam, ab203065, 1 mg/mL) with a concentration of 10 μ g/mL and left overnight at a temperature of 4°C. Antibody dilutions were performed in 5% PBS/BSA.

The next day, the slides were washed two times with PBS-T 0.1% for 5 minutes and incubated for one hour with rabbit secondary antibodies conjugated with HRP. Thereafter, the slides were washed again two times with PBS for 5 minutes. The slides were then incubated in Bright DAB substrate kit. When brown discoloration was seen, the slides were briefly rinsed in demi water to stop the DAB reaction. Next, counterstaining was done with Hematoxylin QS for 10 seconds, followed by a rinse with tap water for 10 minutes. Finally, the slides were dehydrated through 70% ethanol (2 times 1 minute), followed by 96% ethanol (2 times 1 minute), 100% ethanol (2 times 5 minutes), and a coverslip was applied with Pertex.

The results of this first staining showed some positive staining, however there was no staining present in the nucleus of the cells, which is where PAX1 is expected to be located. Because of this, the protocol was adjusted. In the second protocol, prior to the application of 5% PSA/BSA, heat-mediated antigen retrieval was performed, using a 10 mM citrate buffer (pH 6). A water bath was preheated at 37°C and after the slides were incubated, the water bath was turned to 70°C for 30 minutes. After retrieval, the slides were kept outside the water bath for 10 minutes in order to cool down. The samples that were used for this second test contained canine NP, both juvenile (mostly NCs) and adult mixed breed (mostly NPCs).

After application of the protocol explained above, on some slides, staining was present in both nucleus and cytoplasm. Due to excessive staining, the antibodies for the third protocol were more diluted. Now both a concentration of 4 μ g/mL and 2 μ g/mL were used. This staining was done on canine NP, both juvenile and adult mixed breed, and decalcified canine IVD. Results showed that 4 μ g/mL is the optimal concentration for adult mixed breed NP and 10 μ g/mL (from the initial test) is the optimal concentration for juvenile NP. Unfortunately, this protocol did not work on the decalcified canine IVD. Decalcification might alter the immunoreactivity.^{38,39}

However, EDTA was used to decalcify the slides, which is a mild way of decalcification and should not have a significant effect on the immunoreactivity.^{38,39} For this reason, failure of the IVD to stain may be due to a too strong dilution and thus a concentration of 20 μ g/mL was used.

Mixed breed NP was used as a positive control with a concentration of 4 μ g/mL. The protocol above was now tested on alginate beads cultured canine NPCs, with a concentration of 20 μ g/mL, 10 μ g/mL and 4 μ g/mL. This time, a negative control was used as well, using rabbit polyclonal IgG (Abcam, ab37415, 5 mg/mL) with a concentration of 4 μ g/mL. However, in practice, it is found that nonspecific staining is often seen with rabbit IgG. PAX1 antibodies with a concentration of 4 μ g/mL seemed to work on alginate beads cultured canine NPCs.

Eventually, the optimal protocol for PAX1 immunohistochemistry seemed to be as summarized in Table 3.

	Antibody	Concentration in 5% PBS/BSA	Antigen retrieval (Y/N)
Positive control	Abcam, ab203065, 1 mg/mL	4 μg/mL	Y
Negative control	Abcam, ab37415, 5 mg/mL	4 μg/mL	Y
Decalcified canine intervertebral discs	Abcam, ab203065, 1 mg/mL	20 μg/mL	Y
Alginate beads cultured canine nucleus pulposus cells	Abcam, ab203065, 1 mg/mL	4 μg/mL	Y

Table 3 – Concise summary of the immunohistochemistry protocol. PBS = phosphate-buffered saline, BSA = bovine serum albumin, Y = yes, N = no.

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