Evaluation of pain markers expression, nerve fibres ingrowth and establishment of an in vitro model of neurites growth in degenerated intervertebral disc (IVD)

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Major Research Project

MSc Biology of Disease – Biomedical Sciences

2020/2021

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1. Abstract

Low back pain due to the degeneration of the intervertebral disc (IVD) is the most common cause of disability worldwide. Pain is the main debilitating symptom of degenerated IVD, mainly caused by the ingrowth of nociceptive nerve fibres into the deeper layer of the IVD. The production of pain-related neuropeptides by IVD cells is also responsible for the triggering of nociceptive stimuli involved in pain perception. Therefore, it is crucial to investigate the nerve ingrowth processes and the expression of these pain-related markers in degenerative disc disease to determine new strategies for pain treatment. Indeed, it has been shown how the cyclooxygenase-2 (COX-2) inhibitor, celecoxib (CXB) is able to halt degeneration and address IVD-related pain via intradiscal delivery, reducing systemic side effects. In the present study, the presence of nerve fibres and the expression of the neurotrophin NGF in the degenerated IVD were determined in rat and canine models. In addition, the pain-related markers expression was evaluated in vitro in human nucleus pulposus (NP) and annulus fibrosus (AF) cell cultures, upon stimulation with TNFα. Overall, pain marker expression was increased upon proinflammatory stimuli. However after CXB treatment, their expression was increased in a dose-dependent manner, in contrast to what is observed in vivo, suggesting the presence and influence of other factors which play a crucial role in the therapeutical effect of CXB in vivo. Moreover, an in vitro model was established using mouse dorsal root ganglion (DRG) explants cultured in conditioned media of human inflamed IVD cells to evaluate the effects of the latter on neurites growth. Surprisingly, inflamed conditioned media seems to not have any effect on neurites outgrowth, as well as for the CXB-treated one. However, both conditioned media seemed to have a protective effect against neural cell death, resulting in higher number of living neurons. Certainly, follow-up studies are necessary to determine the mechanisms behind pain in the degeneration of intravertebral discs, including neurites ingrowth and neuropeptide related to pain.

2. Layman's summary

Low back pain, due to the degeneration of the intervertebral disc (IVD), the gelatinous structure between the vertebras in the spinal column, it is the most common cause of painful disability worldwide. Pain is the principal and most debilitating symptom of degenerated IVD, mainly caused by the ingrowth of nerve fibres responsible for pain perception into the deeper layer of the IVD. The production of small proteins related to pain by IVD cells is also responsible for the triggering of painful stimuli involved in pain perception. Therefore, it is crucial to investigate the nerve ingrowth and the expression of these pain-related proteins to determine new therapeutical approaches to treat pain at its source. Indeed, it has been shown how the selective anti-inflammatory drug, celecoxib (CXB) is able to treat degeneration and IVD-related pain via intradiscal delivery, reducing generalized side effects. In the present study, the presence of nerve fibres and the expression of the protein NGF, involved in the induction of nerve growth in the degenerated IVD, were determined in rat and canine models. Unfortunately, it has not been possible to determine the nerve ingrowth into the degenerated IVD of the animal models but an increase of NGF expression was observed in the degenerated rat IVD. In addition, the expression of proteins related to pain was evaluated by mean of the culture of two IVD cell types coming from the IVD structures nucleus pulposus (NP) and annulus fibrosus (AF), after being stimulated with a small protein, TNFα, which induce inflammation to the cells, to resemble the degenerative process that happens in the human body. Overall, their expression was increased upon inflammatory stimuli. However, after CXB treatment, which was supposed to reduce their expression as it happens physiologically, unexpectedly the pain-related proteins expression was increased in a way dependent on the drug dosage, suggesting the presence of other factors which play a crucial role in the therapeutical effect of CXB in the human body compared to the cell culture. Moreover, a cell culture model was established by mean of explants of mouse dorsal root ganglion (DRG), a group of neurons and other cell types, cultured in culture media conditioned by human inflamed IVD cells to evaluate the effects of this latter on the neurites growth. Surprisingly, inflamed conditioned media seems not to have any effect on neurites outgrowth, as well as for the CXB-treated one. However, both conditioned media seemed to have a protective effect against neural cell death, resulting in higher number of alive neurons. Certainly, follow-up studies are necessary to determine the mechanism involved in the connection between the degeneration of intravertebral discs and the presence of pain markers, including neurites ingrowth and neuropeptide related to pain.

3. Introduction

3.1 Low back pain

Low back pain (LBP), which affects millions of people, is the main cause of debilitating pain worldwide (Hoy et al. 2015). It affects 80% of the population at certain moment during their life (Binch et al. 2015b). It is responsible for a heavy socioeconomic burden, mainly because of its influence on quality of life (Hoy et al. 2015). Due to aging of the population, bad lifestyles and associated risk factors for IVD degeneration, the prevalence of this condition is therefore predicted to escalate in the near future (Sheng et al. 2017; Tellegen et al. 2018; Rudnik-Jansen et al. 2019). Moreover, genetic predisposition, unhealthy lifestyle and mechanical overload can be contributing and aggravating factors (Battie et al. 2009; Tellegen et al. 2018). Lower back pain is divided in specific or non-specific. Specific back pain is caused by trauma, spinal tumours or infection. On the other hand, non-specific back pain arise in 80–90 % of LBP cases and the underlying cause is still unknown (Takahashi et al. 2008; Binch et al. 2015b). Indeed, between 5% and 10% of all low back pain cases develop into chronic LBP (CLBP) and 40% of these cases are due to intervertebral disc (IVD) degeneration (Luoma et al. 2000; Binch et al. 2015b).

3.2 Intervertebral disc degeneration

The intervertebral disc is characterized by the absence of nerves and vessel in its structure and it consists of three interacting structures: the nucleus pulposus (NP), the gelatinous core of the IVD, the annulus fibrosus (AF), the fibrous ring surrounding the NP, and the end plate (EP), the cartilaginous interface between the bony vertebra and the IVD (Binch et al. 2015a).

During disc degeneration, the structure of the NP and the AF undergo many molecular and morphologic alterations (Antoniou et al. 1996; Rudnik-Jansen et al. 2019) (***Figure 1***). In particular, it is defined by the depletion of extracellular matrix (ECM), especially proteoglycans (PGs), as well as by impaired biomechanical properties due to changing in the disc homeostasis (Liebscher et al. 2011; Le Maitre et al. 2014; Binch et al. 2015a; Rudnik-Jansen et al. 2019). This results in an asymmetry between anabolic and catabolic processes, toward a pro catabolic environment (Le Maitre et al. 2014; Binch et al. 2015a; Rudnik-Jansen et al. 2019).

Accumulating evidence reveals the contribution on a molecular level of pro-inflammatory factors in the degenerative and pain mechanisms (Risbud et al. 2014; Tellegen et al. 2018). In detail, tumour necrosis factor-alpha (TNFα) and interleukin-1 (IL-1) play a big effect within IVD degeneration (Takahashi et al. 1996; Le Maitre et al. 2007; Binche et al. 2015a). These pro-inflammatory cytokines

induce catabolic alterations in the IVD by mean of an overproduction of matrix degrading enzymes, such as matrix metalloproteinases (MMPs), disintergrin and metalloproteinase with thrombospondin motifs (ADAMTS) (La Maitre et al. 2004; Pockert et al. 2009; Binch et al. 2015a; Navone et al. 2017; Tellegen et al. 2018).

This results in a depletion of collagen type II and proteoglycans in the NP, leading to water deficiency, hence impairment of mechanical stress-absorbing properties of the IVD (Freemont et al. 2009; Navone et al. 2017; Tellegen et al. 2018). These biophysical and mechanical alterations impair the motile segment of the spine, affecting the spinal stability and reducing the disc height, with the consequent compression of nerves protruding through the intervertebral foramen (Morgan et al. 1957; Tellegen et al. 2018). Clefts and tears arise in the degenerating AF and ultimately the disc ends up to bulge into the spinal canal, causing the compression of the spinal cord and surrounding nerves (Tellegen et al. 2018). For this reason, the deficit in IVD integrity is an element of concern for eventual spinal aggravations, such as LBP (Morgan et al. 1957; Rudnik-Jansen et al. 2019).

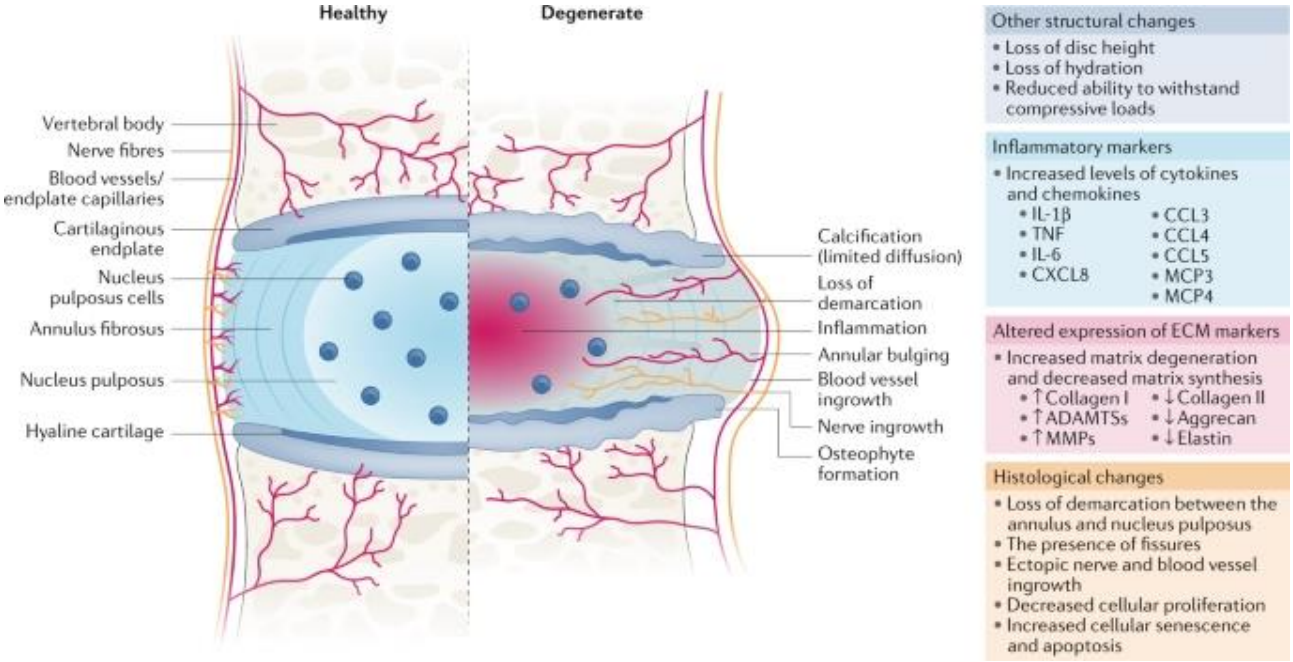


***Figure 1**. Schematic comparison between a healthy and degenerated IVD – Binch et al. (2021)*

4.3 Nociceptive fibres ingrowth in degenerated IVD

As previously said, the IVD is a poorly innervated tissue. However, sensory and sympathetic perivascular nerve fibres innervate roughly 3 mm of the outer structure of the AF (Palmgren et al. 1996; Bogduk et al. 1981; Binch et al. 2015b). The nerve fibres present within the healthy IVD have usually a small diameter, characteristic of nociceptive C-fibres (McCarthy et al. 1991; Binch et al. 2015b). Even though pain is the main clinical symptom in IVD degeneration, the exact mechanism is

still unclear. LBP is commonly attributed to anatomical pain sources, such as IVD or facet joint degeneration, but the roots of the discogenic pain signal in the degenerated IVD is still debated. (Allegri et al. 2016; Rudnik-Jansen et al. 2019).

While back pain is mainly referred to spinal nerves compression by lumbar IVDs bulging and herniation, chronic LBP caused by IVD degeneration is proposed to be aggravated by the ingrowth of nociceptive nerve fibres into the deeper structure of the lumbar IVD (Binch et al. 2015b).

In fact, the degenerating IVD comes to be progressively vascularized by small blood vessels and innervated by sensory nerve fibres, which grow into the deeper structure of the degenerated IVD, possibly eliciting discogenic pain (Risbud et al. 2014; Othori et al. 2015; Binch et al. 2015b; Binch et al. 2015a).

Indeed, tears and fissures in the degenerated AF work as an entry opening for nociceptive nerves and blood vessels growth into the degenerated IVD (Binch et al. 2015b). In addition, these fissures are characterized by a decreased internal pressure, allowing the ingrowth of nerves and blood vessels. On the other side, in healthy IVD the swelling pressure, produced by the NP, prevents its vascularization (Stefanakis et al. 2012; Binch et al. 2015b).

Previous studies demonstrated that nerves are located within the inner AF and NP of degenerated IVDs and, importantly, the correlation was found between nerve ingrowth and painful discs (Freemont et al. 1997; Stefanakis et al. 2012; Binch et al. 2015b). In addition, it was further demonstrated that the ingrowing nerves in the EP co-localised with nerve growth factor (NGF)–positive blood vessels (Freemont et al. 2002; Binch et al. 2015b). This suggests that endothelial cells are the first to grow into the IVD, followed by NGF reactive nerves which grow alongside them. Indeed, it has been found a strong correlation between discreet alterations and innervation of the endplate and showed a link between higher TNF-α expression and nerve ingrowth (Ohtori et al. 2006). Moreover, endplate alterations induce a higher catabolic enzyme production, responsible for matrix molecules degradation, like aggrecan, which has the property to impede neurite ingrowth and endothelial cell infiltration in a healthy IVD (Johnson et al. 2005; Johnson et al. 2006). Catabolic effects on the IVD, in particular proteoglycans loss, is stimulated by growth factors and proinflammatory cytokines, such as IL-1β and TNF-α. Indeed, it has been observed a higher production of matrix metalloproteinase (MMP), besides ADAMTS4, from human NP cells in degenerate IVD and showed the regulation of MMPs by IL-1β in human NP cells (Le Maitre et al. 2004; Le Maitre et al. 2005; Le Maitre et al. 2007b; Hoyland et al. 2008). Therefore, the deficit of proteoglycans due to the increased production of such enzymes from human NP cells could be

responsible for the ingrowth of blood vessels and nerves into the IVD. Aggrecan also exerts its role on disc hydration by trapping water. For this reason, the aggrecan degradation results in disc height decrease and pressure impairments within the NP, which is responsible for extended fissuring in degenerated IVD (Stefanakis et al. 2012). Indeed, nerves present within degenerated IVD mainly colocalise with fissures, which allow facilitated ingrowth into the inner part of the IVD, in correspondence of altered fibrous matrix (Binch et al. 2015b). This is in line with Stefanakis et al. (2012), who showed robust presence of nerves and blood vessels into annular fissures, because of its permissive environment to nerves and blood vessels growth, providing a possible entrance into the inner region of the degenerated IVD (Stefanakis et al. 2012). PGP9.5 has been commonly used as a marker to identify nerves in the IVD tissue (Ohtori et al. 2006; Tolofari et al. 2010; Bailay et al. 2011; Lee et al. 2011). Interestingly, Binch et al. (2015b) observed PGP9.5-positive NP cells as well in human IVD samples. This is the first time that NP cells were found immune-positive for this neural marker. Indeed, Lee et al. (2011) showed a robust positive link between the PGP9.5 and NGF expression within human NP cells. On the other side, Tolofari et al. (2010) did not find any PGP9.5 positive human NP cells within the degenerated IVD. These pieces of evidence of identifying nerve fibres within the IVD using immunohistochemistry are quite contradicting and further studies are necessary. Although the results of PGP9.5 expression in NP cells are contradicting, nerve ingrowth of PGP9.5 positive fibres is found in severely degenerated IVDs (Ashton et al. 1994; Brown et al. 1997; Ohtori et al. 2006; Ozawa et al. 2006; Tolofari et al. 2010; Lee et al. 2011). However, it is necessary to clear the role of the chemoattractive signal represented by neurotrophines in stimulating and guiding nerve growth into the degenerated IVD.

4.5 Neurotrophines and neuropeptides production in degenerated IVD

Recent studies focusing on the underlying mechanisms of low back pain related to neural ingrowth in degenerated IVD have shown that NP cells increment their expression of neurotrophines and angiogenic factors upon mechanical injury (Alkhatib et al. 2014), strain (Gawri et al. 2014) and pro-inflammatory cytokines, such as TNFα, IL1b and IL6 (Purmessur et al. 2008; Binch et al. 2014; Krock et al. 2014).

In particular, Krock et al. (2014) recently proved a substantially higher nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) expression by IVD organ cultures from degenerated IVD in comparison to those from healthy ones (Binch et al. 2014).

The expression of these molecules seems to promote the survival and ingrowth of nociceptive sensory nerves into the degenerate IVD (Binch et al. 2015b). On the other hand, in the healthy IVD,

many repulsive molecules are produced, such as aggrecan (Johnson et al. 2005; Johnson et al. 2006), chondromodulin (Takao et al. 2000) and semaphorins (Tolofari et al. 2010), which inhibit nerve and endothelial cell ingrowth, (Binch et al. 2015b). However, the production of these molecules is reduced during the IVD degeneration, allowing free entry into the IVD (Binch et al. 2015b).

Therefore, these neurotrophic factors, which promote peripheral nociceptive sensory neurons to grow into the degenerated IVD, are responsible for sensitizing the area to pain, making the degenerated IVD painful (Freemont et al. 1997; Binch et al. 2014; Krock et al. 2014; Rudnik-Jansen et al. 2019).

NGF and BDNF exert their effect by binding to two distinct receptors: tropomyosin-related kinase A (TrkA) and B (TrkB) respectively. In addition, both of them bind with a lower affinity to p75 neurotrophin receptor (p75NTR) (Chao et al. 1995; Fayard et al. 2005; Orita et al. 2010). In particular, TrkA located at the peripheral nerve ending plays a role in the formation of the NGF-TrkA complex, which is retrogradely transported to the dorsal root ganglion (DRG) soma, where it induces the expression of SP and CGRP (Ehlers et al. 1995; Orita et al. 2010). Hence, degenerated IVD with a higher expression of neurotrophines and nerve fibres expressing their specific receptors is responsible for the activation of primary afferent neurons, generating discogenic pain (Woolf et al. 1994; Burke et al. 2002; Orita et al. 2010).

Moreover, Brown et al. (1997) showed that ingrowing nerves in the degenerated IVD produce small inflammatory pain-related neuropeptides responsible for nociception, such as substance P (SP) and calcitonin gene-related peptide (CGRP), which are able to trigger the surrounding nerves and produce painful stimuli. Also Coppes et al. (1997) found nerve fibres producing substance P protruding into the internal AF. Moreover, these nociceptive c-fibres are seen to be NGF-dependent for their depolarization and survival (Averill et al. 1995; Snider et al. 1998; Orita et al. 2010).

Indeed, it has been proved that also NP cells express the sensory peptides, substance P and CGRP, which can be upregulated in response to proinflammatory stimulation (Binch et al. 2014). Hence, an increased expression of these sensitising molecules for nerve endings makes mechanical stress, which is commonly irrelevant to the disc nociceptors, responsible for pain perception. (Binch et al. 2015b).

The neuropeptide SP is a neuropeptide generally involved in pain, by stimulating the nociceptive c-fibres via NK1 receptor, implicating a possible role in pain sensitization of the degenerate IVD (Binch et al. 2014). Whereas, CGRP causes hyperalgesia via both protein kinase A and C pathways, which

implies that also high CGRP production could induce pain stimuli in the peripheral nerves (Sun et al. 2004; Orita et al. 2010).

Additionally, in the degenerated IVD, the ingrowth of nociceptive nerve endings is also shown to be guided by the pro-inflammatory mediator cyclooxygenase-2 (COX-2)-derived prostaglandin E2 (PGE_2), which could sustain to the discogenic pain onsetting (Simon et al. 2014; Tellegen et al. 2018). In fact, it has been proven that PGE_2 enhances the neurites number, a morphological characteristic of neuronal differentiation (Hiruma et al. 2000; Mitani et al. 2016; Nango et al. 2017). Indeed, PGE_2 is thought to promote neuronal differentiation via activation of the prostanoid receptor-mediated signaling pathway (Nango et al. 2017).

Therefore, PGE_2 plays a role in inflammation and pain, which derives from the effect of PGE_2 on peripheral sensory neurons, causing hyperalgesia due to principally via EP1 receptor signaling (Funk et al. 2001; Moryiama et al. 2005; Ricciotti e FitzGerald 2011).

4.9 Anti-inflammatory drugs as pain relieving strategies

Since pain related to IVD degeneration is exacerbated by inflammation, LBP is usually treated with anti-inflammatory drugs, such as corticosteroids or nonsteroidal drugs, which are commonly administered orally or injected locally, in order to reduce inflammation and pain (Alimasi et al. 2013; Tellegen et al. 2018; Rudnik-Jansen et al. 2019).

Despite corticosteroids are mainly used for their powerful anti-inflammatory properties, their long-term use implies adverse side effects, such as severe osteoporosis and risk of gastrointestinal problems (Lukert et al. 1990; Rehman et al. 2002; Lian et al. 2005; Rudnik-Jansen et al. 2019).

Therefore, a safer solution is represented by nonsteroidal anti-inflammatory drugs (NSAIDs). In particular, they block the activity of COX-1 and/or COX-2, inhibiting the synthesis of the inflammatory-mediator PGE_2 (Alimasi et al. 2013). Among the NSAIDs, selective COX-2 inhibitors are preferred for their effectiveness on acute pain, causing fewer gastrointestinal side effects (Shi et al. 2008; Alimasi et al. 2013).

Moreover, these treatments are challenged by the limited tissue diffusion and bioavailability of orally administered drugs into the avascular IVD (Motaghinasab et al. 2014; Zhang et al. 2014; Tellegen et al. 2018). Hence, local delivery of anti-inflammatory drugs might be a more effective approach for pain treatment in symptomatic IVD degeneration (Tellegen et al. 2018). Indeed, intradiscal injections can decrease the risk of systemic side effects and might represent an alternative strategy compared to a more invasive surgery (Cao et al. 2011; Rudnik-Jansen et al. 2019).

In addition, drug delivery systems with high drug loading and sustained drug release over prolonged period of time seem to allow to have local high doses of the drug for longer period, therefore increasing efficacy and minimizing injections. Hence, drug delivery systems allowing safer, localized and prolonged exposure to drugs are an attractive approach to reduce long term LBP (Tellegen et al. 2018; Rudnik-Jansen et al. 2019).

4.10 Drug-delivery systems

To this end, resorbable and degradable polymers showed a good regulation over degradation and release properties, in addition to biocompatibility. In particular, microspheres composed by biodegradable polyesteramide (PEA) polymers proved to be a valuable biomaterial system for local drug delivery (Andre-Guerrero et al. 2015). The advantage of these polymers is that they mainly degrade via an enzymatic mechanism, leading to a constant drug release in the degenerated IVD environment, due to their unique surface erosion kinetics (Tellegen et al. 2018). PEA microspheres (PEAMs) can then be considered as an autoregulatory drug delivery system (Janssen et al. 2016). In fact, in the inflammatory environment of degenerated IVD, abundant proteases are responsible for an increased microsphere degradation and therefor higher drug release (Le Maitre et al. 2007). In a recent study, PEAMs were proved to be safe for intradiscal administration in a canine model of degenerated IVD (Willems et al. 2015; Tellegen et al. 2018). Physiological IVD degeneration in dog mimics IVD degeneration in humans with comparable molecular, histological and clinical characteristics, therefore the dog is a valuable translational large animal model to study chronic lower back pain (Bergknut et al. 2012; Bach et al. 2014).

Recently, a study assessed the local delivery of PEA microspheres loaded with the COX-2 inhibitor, celecoxib (CXB), using a clinically relevant canine model. It has been demonstrated that robust release of CXB from PEA microspheres strongly suppressed the PGE_2 synthesis, inhibiting IVD degeneration (Tellegen et al. 2018). In fact, intradiscal injection of celecoxib-loaded PEA microspheres ameliorated the overall histological grade of IVD, besides the fact that the decrease of PGE_2 levels was also accompanied by a decrease in NGF expression (Tellegen et al. 2018). Therefore, the reduction of local PGE_2 levels and NFG immunopositivity, in response to local delivery of the celecoxib-loaded PEAMs, prove encouraging beneficial effects for chronic back pain due to IVD degeneration. Indeed, local delivery of CXB-PEAMs also inhibited glycosaminoglycans (GAG) depletion and halted the degeneration within the NP, preserving the water load and collagen integrity (Tellegen et al. 2018). The effect of this approach is dual: by reducing inflammation and thus decreasing the amount of pain mediators and by inhibiting proteoglycan degradation (Tellegen

et al. 2018). In fact, both these mechanisms inhibit the ingrowth of nerves and blood vessels into the IVD (Purmessur et al. 2015; Navone et al. 2017). These results prove that celecoxib-loaded PEA microspheres system might be very promising to treat chronic back pain due to IVD degeneration. In fact, celecoxib loaded on PEAMs was safely injected in the canine IVD and there it exercised anti-inflammatory and anti-degenerative effects (Tellegen et al. 2018). This evidence implies that local robust release of celecoxib decreases inflammation and, by reducing pain mediators such as PGE_2 and NGF, might also alleviate chronic back pain (Tellegen et al. 2018). All in all, although pain is a severe symptom in LBP, the biochemical mechanism behind nerve ingrowth and nociception in IVD degeneration is poorly understood. Additionally, the action mechanism of CXB in reducing pain in IVD degeneration needs to be further elucidated.

Therefore, the aim of this study was to assess the expression of pain-related markers, in particular the neurotrophines NGF, BDNF and the neuropeptides CGRP, SP involved in pain sensitization and their role in neurites ingrowth in degenerated IVD.

Moreover, we wanted to investigate the effect of celecoxib on the before mentioned pain-related markers and consequentially on nociceptive neurite outgrowth.

In addition, this study established a first primordial in vitro model of inflamed human IVD cells and neurite outgrowth, which could be useful for further future comprehension of the mechanisms involved in the pain source of the degenerated IVD and lower back pain.

5. Materials and methods

5.1 Rat IVD sectioning

In brief, 4 rats (6 weeks old) underwent an anterior annulus puncture (AAP) on their L4-L5 and L5-L6 IVDs (Bert Joosten's lab, Maastricht University). Other 4 rats (6 weeks old) were used as control with no surgery performed. For both groups, the IVD L3-L4 was additionally used as control. 8 weeks post AAP procedure the rats were terminated. After termination, discs were isolated, decalcified and embedded in paraffin. Subsequently, the IVD samples were cut along the sagittal plane in ventral-dorsal direction using a microtome (Leica). For each IVD, 5 µm and 10 µm thick sections were obtained. In both cases, the sections were cut at 3 different depths of the IVD structure, 200 µm apart from each other (***Figure 2***).

For an extra fixation of the sections on positively charged slides, they were left to dry overnight at RT before being placed for 8 hours on a 60°C plate and subsequently baked at 60°C overnight before the histological and immunohistochemistry procedures.

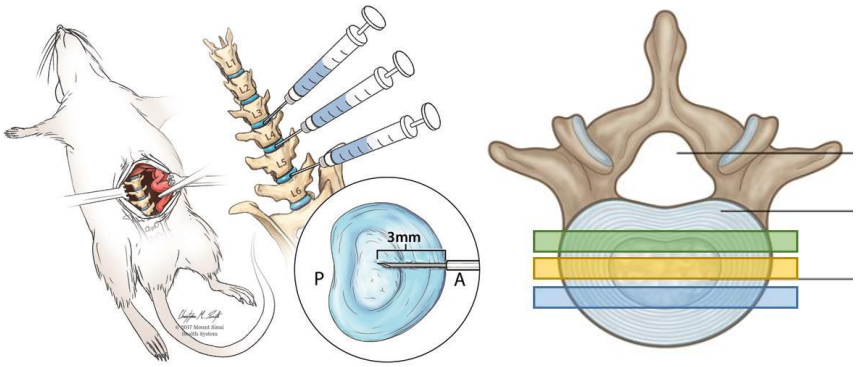


Figure 2*. IVD degeneration induction procedure and cutting scheme and orientation of IVD samples - Image created from Mount Sinai Health System - Evashwick-Rogler et al. (2018)*

5.2 Canine IVD sectioning

In brief, 3 dogs (19 months old) underwent an IVD degeneration induction procedure at five alternating levels (T12-T13, L1-L2, L3-L4, L5-L6 and L7-S1) (Tellegen et al. 2018). 4 weeks post induction, CXB loaded and empty PEAMs were injected at low dose (LD) and high dose (HD) in the intervertebral discs. 12 weeks post intervertebral injection the dogs were terminated. After termination, discs were isolated, decalcified and embedded in paraffin. The same cutting and fixation method and approach were followed as those used in the before-mentioned rat IVD sectioning (***Figure 2.*** IVD degeneration induction procedure and cutting scheme and orientation of IVD samples).

5.3 Haematoxylin staining

The haematoxylin staining was performed on both the 5 µm rat and canine IVDs sections to investigate the morphology of the different IVD structures. After having deparaffinized the sections, they were stained with Wiegert's haematoxylin solution. Subsequently, the sections were stained with eosin solution, ready for the degeneration grade evaluation.

5.4 Safranin-O/Fast Green staining

The safranin-O/ fast green staining was performed on the 5 µm rat IVDs sections to investigate the structure of the IVDs, since different cartilaginous structures stain differently. After having deparaffinized the sections, they were stained with Wiegert's haematoxylin solution, after this step, they were stained with 0.4% aqueous Fast Green. Then, the sections were stained with 0.125% aqueous Safranin-O solution, ready for the degeneration grade evaluation.

5.5 Degeneration scoring of rat IVD sections

The sections of rat IVD stained with haematoxylin and safranin-O/fast green were scored to assess the respective degeneration grade, following the scoring system descried by Evashwick-Rogler et al. (2018) (***Figure 3***), which takes into account 5 different IVD parameters: annulus fibrosus morphology, border between AF and NP, cellularity of nucleus pulposus, matrix of NP and EP structure. Each parameter ranged from 0, healthy IVD, to 2, degenerated IVD. The scoring was performed in a blind and random order by two evaluators using the validated histological grading system (***Figure 3***), using an Olympus BX41 microscope. The significance tests were performed by mean of t-test.

TABLE 1 Intervertebral disc (IVD) degeneration grading scale[a]

Annulus fibrosus		
Grade	0	Normal, pattern of fibrocartilage lamellae (U-shaped in the posterior aspect and slightly convex in the anterior aspect) without ruptured fibers and without a serpentine appearance anywhere within the annulus
	1	Ruptured or serpentine pattern fibers in less than 30% of the annulus
	2	Ruptured or serpentine pattern fibers in greater than 30% of the annulus
Border between annulus fibrosus and nucleus pulposus		
Grade	0	Normal
	1	Minimally interrupted
	2	Moderate/severe interruption
Cellularity of nucleus pulposus		
Grade	0	Normal cellularity with large vacuoles in the gelatinous structure of the matrix
	1	Slight decrease in the number of cells and fewer vacuoles
	2	Moderate/severe decrease (>50%) in the number of cells and no vacuoles
Matrix of nucleus pulposus		
Grade	0	Normal gelatinous appearance
	1	Slight condensation of the extracellular matrix
	2	Moderate/severe condensation of the extracellular matrix
Endplate		
Grade	0	Homogenous structure; regular thickness
	1	Slight irregularity with limited number of microfractures; locally decreased thickness
	2	Severe irregularity with multiple microfractures of endplate; generalized decrease of thickness

***Figure 3**. Intervertebral disc degeneration grading scale - Evashwick-Rogler et al. (2018)*

5.6 NGF and PGP9.5 immunohistochemistry and cellularity analysis

The immunohistochemistry against NGF was performed on rat IVD sections and against PGP9.5 both on rat and canine IVD sections. In particular, the rabbit antibody anti-NGF (ab52918, Abcam) at 1:3000 dilution (0.887 ug/mL) was used on 5 µm rat IVD sections. On the other hand, the antibody mouse anti-PGP9.5 (ab8189, Abcam) at 1:400 dilution (2.5 ug/mL) was used on 10 µm rat IVD sections and on 5 µm canine IVD sections. As a positive control, rat kidney 5 µm sections for NGF and rat and dog skin 10 µm sections for PGP9.5 were used. The samples were deparaffinized and then extra fixed in 4% formalin for 25 min. This was followed by the antigen retrieval with citrate buffer (pH 6.0) in water bath at 90°C for 20 min. After washing, the samples underwent the peroxidase blocking step with 0.3% H_2O_2 for 20 min followed by blocking with 5% goat serum and 5% BSA in PBS for 1 hour at RT. The primary antibody was added and incubated at 4°C ON. For the negative control, rabbit and mouse IgG antibodies (Agilent) were respectively used. After this step,

the sections were washed with PBS-Tween and the 1 step detection secondary antibody-HRP system anti-rabbit (100321, BrightVision, ImmunoLogic) for NGF and anti-mouse (160919, BrightVision, ImmunoLogic) for PGP9.5 were used and incubated for 1 hour at RT. After having washed with PBS-Tween, the sections were incubated with the DAB solution (11246742, Chromogen system, Liquid DAB+ Substrate, Dako) The staining was stopped by briefly washing of the sections in milliQ water and then in demi-water, followed by counterstaining with Mayer's haematoxylin, ,dehydration and mounting with mounting media (Depex).

Images of the immunohistological results were taken by mean of Olympus BX41 microscope and the image analyses were conducted using the ImageJ software. The NGF expression in each IVD section was determined by taking pictures of three different positions of each IVD structure (NP, AF, EP), for three replicates. Then for each picture, the NGF expression was evaluated by the software calculating the DAB signal, in terms of positive pixels. The number of cells of the three structures (NP, AF, EP) of the rat IVD sections was determined by the ImageJ software, counting the nuclei stained by haematoxylin.

Then the ratio between NGF signal and cell number was calculated for each structure of each IVD sections and then the mean of the technical replicates was calculated. Moreover, the signal intensity was also determined by the software, which was used to evaluate the ratio between the signal intensity and the stained area for each structure of each IVD sample. All the significance tests were performed by mean of the t-test.

Afterwards, the correlation between the cellularity of each structure and the AAP procedure, and the healthy (score =< 3) and degenerated (score > 3) categories were determined. The significant tests were performed by mean of the t-test. In addition, the correlation between the cellularity of each structure and the IVD degeneration score was determined by mean of the Pearson correlation test.

Indeed, the statistical analysis for PGP9.5 expression were not possible since there was no DAB signal upon the immunohistochemistry.

5.8 Human NP and AF cell culture

Two human spine samples were collected from the University Medical Centre morgue, accordingly to the medical and ethical protocols of the UMC. The cell isolation was performed on two of the lower lumbar IVDs. The spine samples were washed with water and ethanol 70% and cleaned from fat and excessive tissues. Then they were washed again. After that, an incision was performed in

correspondence of the IVD, along the endplate, by mean of a scalpel. At this point the IVD was disconnected form the vertebra and a NP and AF sample were collected separately. At this point the tissue samples were washed with DMEM and minced using a scalpel. The first digestion was performed in 0.004% DNase and 0.2% pronase in DMEM for 1 hr at 37°C on a tube roller. Then the digested tissues were centrifuged for 2 min at 1750 rpm. After removing the first digestion solution, the second digestion was performed by using 0.05% collagenase and 0.004% DNase in DMEM for NP tissue, and 0.1% collagenase and 0.004% DNase in DMEM for AF tissue overnight at 37°C on a tube roller. The digested tissues were pipetted through a 70 µm cell strainer and centrifugated for 3 min at 1750 rpm. Then they were washed with DMEM and centrifuged again for 3 min at 1750 rpm. The cell pellets were resuspended in expansion media (DMEM 10% FBS, 1% P/S, 1% ASAP, 2.2 mg/mL NaCl), the cell number was counted and diluted to 3000-4500 cell/cm^2 in a flask. Then the cells were cultured for at least one passage and frozen at -80°C.

5.9 Effect of celecoxib on expression of neurotrophines and neuropeptides

The NP and AF cells were seeded in a 12-well plate at 50000 cells/cm^2 density (200000 cells/well) in chondrogenic media (DMEM 2% HAS, 2% ITS-X, 1% PS, 1% ASAP) and incubated ON. The day after the culture media was replaced with expansion media containing 0, 1, 10 or 100 uM celecoxib (LC Laboratory) and 0 or 10 ng/mL of TNFα (DDHB0319091, R&D systems) (***Figure 4***). For a part of the experiment, the expansion media contained also 0 or 10 uM of PGE_2 (P0409, Sigma-Aldrich) (***Figure 5***). Then the cells were incubated for 24 hours. After the incubation period the media was collected for the ELISA and the cells were harvested to perform the qPCR and the Western blot. For a part of the experiment, after the 24 hours of incubation, the condition media was refreshed with expansion media and incubated for 48 hours (***Figure 4***). Subsequently, the conditioned media was collected to perform an ELISA and a qPCR and the neurite outgrowth assay with mouse DRG explants. For each condition 3 replicates were performed.

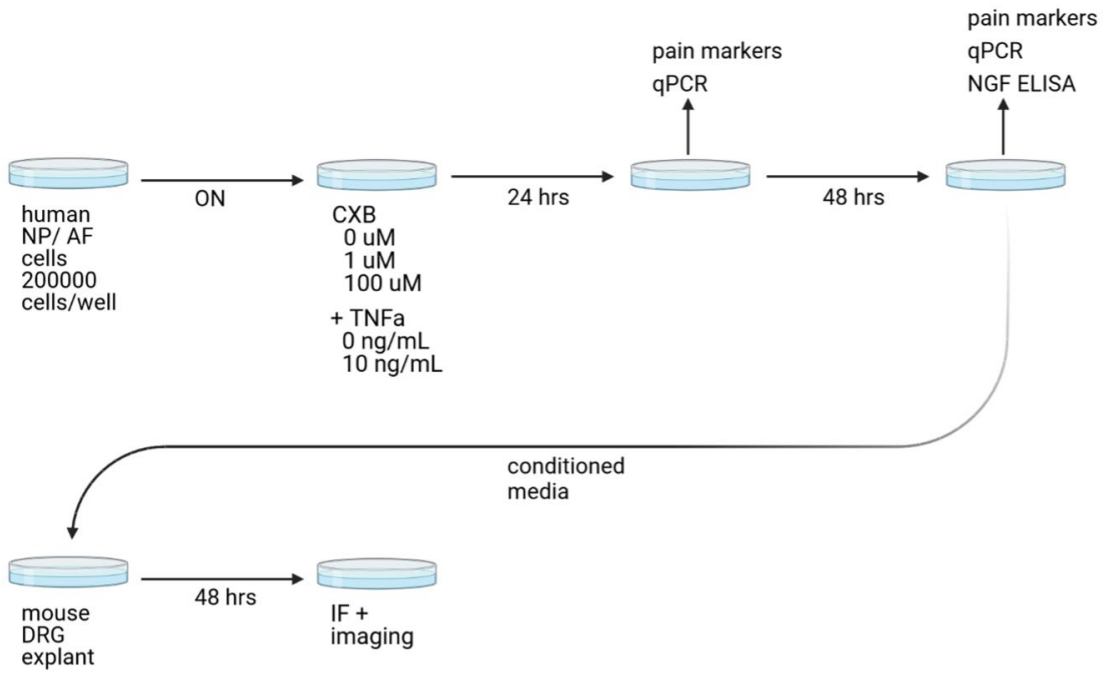


Figure 4. *Experimental set up to investigate the effect of the inflammatory stimulation by TNFα and the therapeutic effect of celecoxib on NP and AF cells after 24 and 72 hours of incubation.*

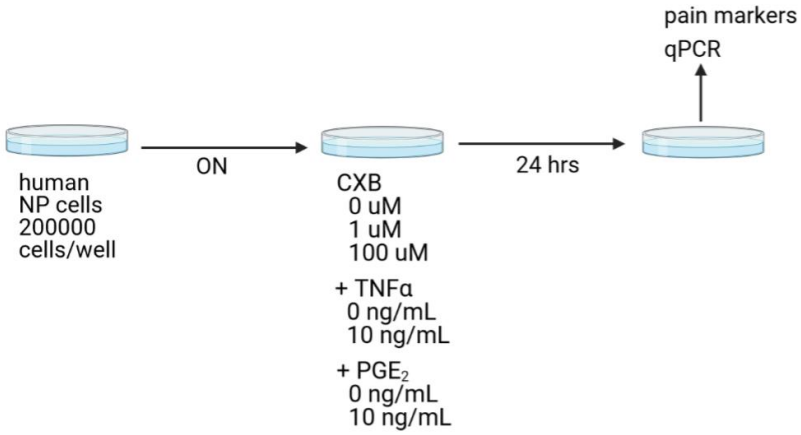


Figure 5. *Experimental set up to investigate the effect of the inflammatory stimulation by TNFα, the therapeutic effect of celecoxib and the eventual reversing effect of PGE_2 on NP cells after 24 hours of incubation.*

5.11 RNA isolation and Real time-PCR analysis

In order to extract the RNA from the NP and AF cells which was used in the real-time PCR, the Trizol-chloroform method was used. To precipitate the suspended RNA, glycogen (00902783, Thermoscientific) in isopropanol was used and centrifuged. The precipitated RNA formed a gel-like pellet on the side-bottom of the tube. At this point the pellet was washed with 75% ethanol and resuspended in RNase-free water. The RNA concentration of the samples was measured using the Nanodrop (260 nm) and then the tubes were stored at -80°C until further use.

To evaluate the gene expression in NP and AF cells upon inflammation induction and celecoxib treatment, RT-qPCR was performed. The cDNA was synthetized from the previously isolated RNA, using the High-Capacity cDNA Reverse Transcription Kit (00587287,Thermo Fisher Scientific), accordingly to the manufacturer's instructions and the T100 Thermal Cycler (BioRad). The RNA starting concentration was 500 ng per reaction. The total volume of the reaction was 20 ul. The cDNA was diluted 5 times in nuclease-free water. The primers sequences were designed using the Primer-BLAST tool of the NIH website and the annealing temperatures was determined empirically (**Table 1**).

Gene	**Primer**	**Sequence**	**Annealing T (°C)**	**Amplicon size (bp)**
NGF	Forward	TGTGGGTTGGGGATAAGACCA	61	140
	Reverse	GCTGTCAACGGGATTTGGGT		
BDNF	Forward	CTACGAGACCAAGTGCAATCC	62	147
	Reverse	AATCGCCAGCCAATTCTCTTT		
CGRP	Forward	TCTAAGCGGTGCGGTAATCTG	60	85
	Reverse	CAGTTTGGGGGAACGTGTGA		
SP	Forward	TGATCTGAATTACTGGTCCGACT	60	60
	Reverse	TCCGGCAGTTCCTCCTTGA		
GAPDH	Forward	TGCACCACCAACTGCTTAGC	60	87
	Reverse	GGCATGGACTGTGGTCATGAG		
RPL19	Forward	ATGAGTATGCTCAGGCTTCAG	60	150
	Reverse	GATCAGCCCATCTTTGATGAG		

Table 1. *Primers' characteristics for the genes of interest NGF, BDNF, CGRP, SP and as control, the housekeeping genes GAPDH and RPL19.*

RT-qPCR was performed using iTaq Universal SYBR Green Supermix (64410722, BioRad), forward and reverse primers (Invitrogen Termo Fisher Scientific) and RNase-free water and the Lightcycler96 (Roche) and CFX96 Real-Time System (BioRad). To determine the relative gene expression, the Pfaffl method was used (Pfaffl M.W 2001). Two reference genes, GAPDH and RPL19, were used to normalize the gene expression of the target genes.

To assess the quality of the amplicons of interest and the absence of primer-dimers, a primer-dimer test was performed after the RT-qPCR. 3% agarose gel was prepared in TAE 1X with 0.1 uL/mL SyberSafe. Loading dye 6X was added to the PCR samples and then transferred to the gel wells. The gel was run at 110V for 60 min and then it was visualized under UV light.

5.12 Western blot

To evaluate the NGF production by NP and AF cell, the Western blot was performed. The samples were washed with ice-cold PBS0. Then the RIPA lysis buffer (GR3378961, Abcam) was added. The cells were scrapped using a pipette tip. At this point, the lysate was centrifuged at 12000 rcf at 4°C for 20 min. The protein concentration was determined by using the BCA protein kit (ThermoScientific). To denaturate the proteins, loading buffer (C31152008, GenScript) was added to the protein lysate and incubated at 95°C for 5 min. At this point the samples were ready for the Western blot. 10 ug of protein per sample were loaded in each well. The gel was run at 100 mV for 1h 30m, transferred to the blot and it was run at 100 mV for 1h 30m. After this step, the membrane was washed with PBS-T and blocked with 5% non-fat dry milk in PBS at RT for 1 hour and then washed again with PBS-T. At this point, the primary antibodies rabbit anti-NGF (ab52918, Abcam) at 1:500 dilution (5.32 µg/mL) and mouse anti-y-tubulin (T6557, SIGMA) at 1:3000 dilution (1 µg/ml) were prepared in 5% non-fat dry milk in PBS-T and incubated ON at 4°C. Then the membrane was washed with PBS-T for 30 min. At this point the secondary antibodies were prepared; mouse anti-rabbit HRP (Agilent) and goat anti-mouse HRP (Agilent) at 1:2000 dilution (5 µg/ml) in 5% non-fat dry milk in PBS and incubated for 1 h at RT. Then the membrane was washed again with PBS-T for 30 min and incubated with SuperSignal West Femto Maximum (ThermoFischer) and imaged with the Imager (BioRad).

5.13 Enzyme-linked immunosorbent assay

The amount of secreted NGF by NP and AF cells in the culture media upon TNFα stimulation and CXB treatment was determined using the DuoSet ELISA kit for Human β-NGF (DY256, R&D systems) and following the manufacturer's instructions. After having coated the plate with the capture antibody it was blocked with reagent diluent (1% BSA in PBS). Then the media samples or standards in reagent diluent were added and incubated for 2 hours at room temperature. After that, the plate was washed and the detection antibody anti-NGF was added, followed by the Streptavidin-HRP solution. Then the substrate solution was added and stopped the reaction with 2N H_2SO_4. In the end, the optical density was determined using a microplate reader set to 450 nm and 540 nm.

5.14 Mouse dorsal root ganglion (DRG) explant culture and outgrowth assay

The mouse dorsal root ganglion explants were provided by Niels Eijkerkamp's Laboratory (University Medical Centre - UMC), which isolated, cultured and processed them. The DRG explants were isolated from the spinal cord of one male mouse and they were plated on poly-L lysine and laminin

coated coverslips. The explants after being processed with dissection medium (HBSS w/o $Ca^{2+}Mg^{2+}$, 5mM HEPES (Gibco, 15630-049), 10mM glucose and trituration medium (Dulbecco's Modified Eagle's Media (DMEM), 10% Heat-Inactivated Foetal Bovine Serum (F9665, Sigma), 1% Pen/Strep) were left to settle for 1 hour in the incubator at 37 °C and then incubate for 48 hours before being treated. To determine the effect of the condition media, coming from the inflamed NP and AF cells and treated with celecoxib, on the DRG cellularity and neurites outgrowth, the dorsal root ganglion explants were then cultured in conditioned media for 48 hours. Subsequently, the DRGs were harvested and processed to perform an immunofluorescence on them (***Figure 4***).

To evaluate the neurites length and cell number of the DRG explants, an immunofluorescence was performed on them to stain the neural markers TUBB3, a cytoskeletal marker, and NeuN, a nuclear marker. The explants were fixed with 4% PFA for 10 minutes then washed with PBS-T. Then they were blocked with blocking solution (5% NDS and 1% BSA in PBS-T) to block non-specific binding and incubate for 60 minutes. After that, the primary antibody anti-beta III Tubulin antibody Rabbit poly (ab18207; 1:2000) and anti-NeuN mouse monoclonal (MAB377: 1:500) in 1% BSA-PBST were added and incubated ON at 4 °C. After being washed, the explants were incubated with secondary antibody; donkey anti rabbit AlexaFluor 488 (1:1000) and donkey anti-mouse AlexaFluor 594 (1:1000) in 1% BSA for 1 hour at RT in the dark. Then washed again and the DAPI solution was added and incubated for 5 min at RT. After being washed, the samples were mounted with Fluorsave and coverslip.

The pictures of the samples were taken by using the confocal microscope (Leica SP8). For each DRG explant, three pictures in three different locations were taken. Image analyses were conducted using the ImageJ software and the plug in Neuralmetrics, which measured the number of neurons, nuclei and the average neurites length. All the significance tests were performed by mean of ANOVA test.

6. Results

6.1 Pain-related markers expression and nerves growth within rat degenerated IVD sections

6.1.1 Rat IVD degeneration scoring

To determine the degeneration grade of the IVD, the degeneration scoring system was used. For the rat IVD sections the degeneration score did not correlate significantly with the procedure which the rat groups underwent, either AAP (n = 8) or Control (n = 16) (***Figure 6***).

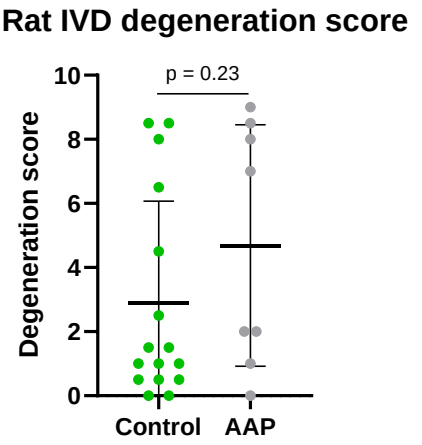


Figure 6*. Scoring of the degeneration grade of the rat IVDs and correlation with the procedure that the mice underwent (Control = no surgery, n = 16; AAP = Anterior Annulus Puncture, n = 8) (p> 0.05).*

The NP cellularity was not significantly lower in the AAP group compared to the Control group (***Figure 7A***). The same trend is true for the AF cellularity (***Figure 7B***). On the other side, the EP cellularity is significantly lower in AAP group compared to the Control group (***Figure 7C***).

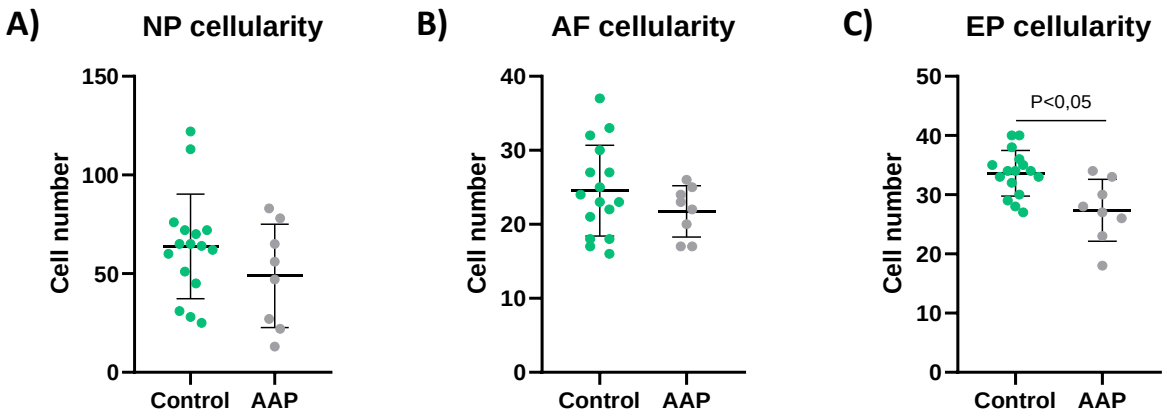


Figure 7*. Cellularity difference between Control and AAP group in NP (A, p> 0.05), in AF (B, p> 0.05) and in EP (C, p< 0.005).*

However, the NP and EP cellularity significantly decreased with IVD degeneration grade (***Figure 8A; 8C***). This was not true for the AF structure which did not show a significant Pearson correlation between the cell number and the degeneration grade of the IVD (***Figure 8B***).

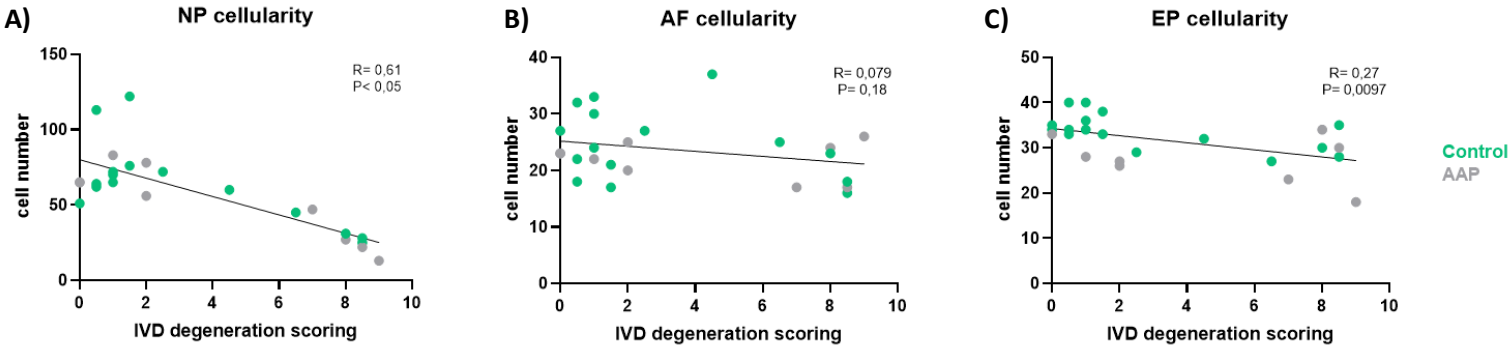


Figure 8. Cellularity difference in NP (A, R> 0.5, P< 0.05), in AF (B, R< 0.1, P> 0.05) and in EP (C, R< 0.5, P< 0.05), accordingly to the IVD degeneration grade.

Subsequently, the IVD samples were grouped by the IVD degeneration score and not by the procedure Control or AAP procedure, assuming a score lower or equal to 3 as healthy (n = 15) (***Figure 10***) and higher than 3.0 as degenerated (n = 9) *(**Figure 11***). In this case, the correlation between a lower cell number and higher degeneration grade of the IVD is more evident.

The NP cellularity significantly decreased in degenerated group compared to the healthy one as expected since NP cellularity is a parameter for scoring, therefore they're interconnected (***Figure 9A***). However, the same decrease is seen for EP cellularity (***Figure 9B***), but not for the AF (***Figure 9C***).

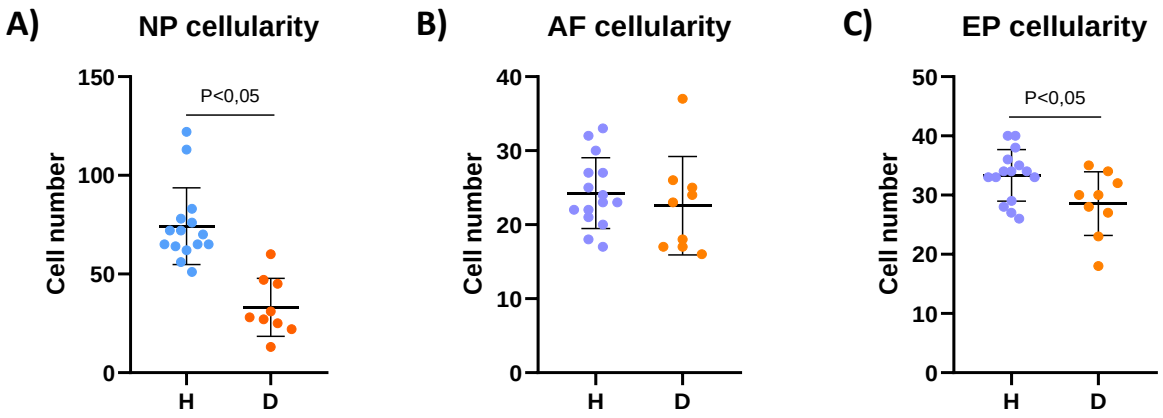


Figure 9. Cellularity difference between healthy (H) and degenerated (D) IVD group in NP (A, p< 0.005), in AF (B, p> 0.05) and in EP (C, p< 0.05). Healthy category: IVD degeneration scoring =< 3.0; Degenerated category: IVD degeneration scoring > 3.0.

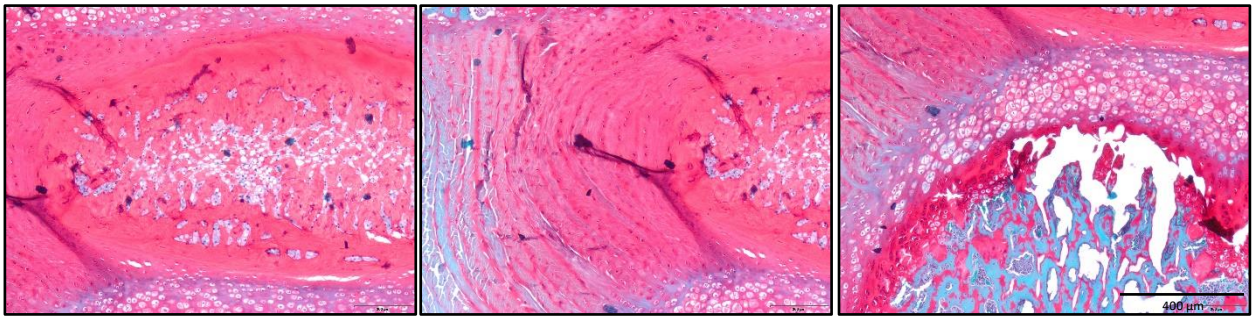


***Figure 10**. Safranin-0/Fast green staining of 5 um healthy rat IVD section. NP (A), AF (B) and EP (C) structures.*

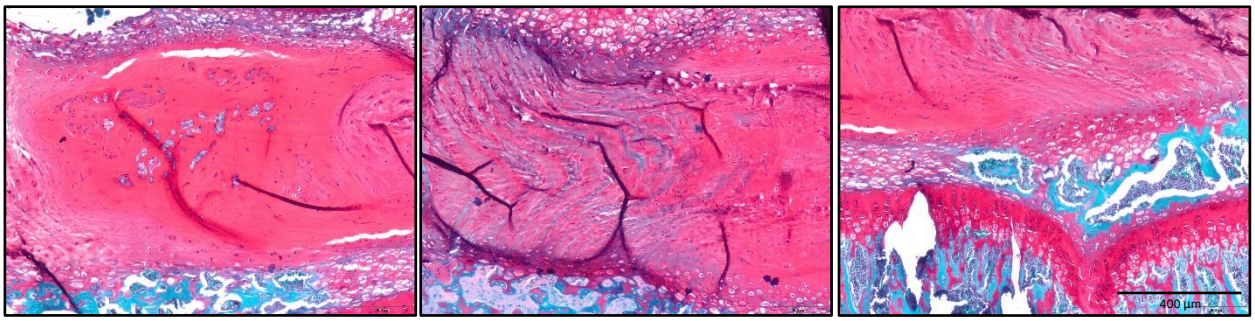


***Figure 11.** Safranin-0/Fast green staining of 5 um degenerated rat IVD section. NP (A), AF (B) and EP (C) structures.*

6.1.2 NGF expression in rat IVD

In order to evaluate the expression of NGF in the degenerated rat IVD model, the immunohistochemistry against NGF was performed (***Figure 14; 15***). The ratio between NGF signal and cell number in the NP structure was shown to be lower in the degenerated group compared to the healthy one but not in a significantly way (***Figure 12A***). On the other side, in the AF, the degenerated group shows a significantly higher expression compared to the healthy one (***Figure 12B***), as well as in the EP structure, although not in significant way (***Figure 12C***).

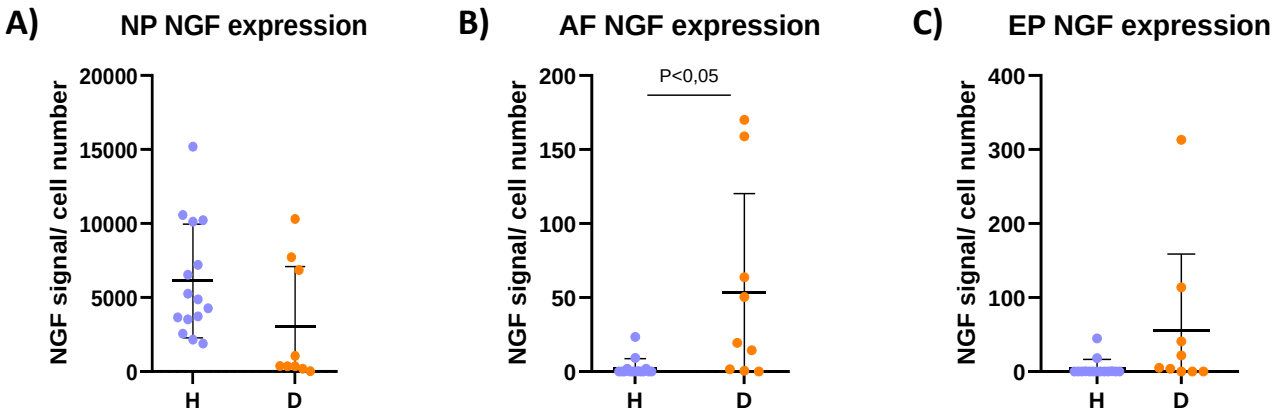


***Figure 12**. Difference in NGF expression and cell number ratio between healthy and degenerated IVD group in NP (A, p> 0.05), in AF (B, p> 0.005) and in EP (C, p> 0.05).*

Instead, the ratio between the NGF signal intensity and the stained area had an opposite trend compared to the previous results. In the NP structure the degenerated group presents a significant

higher value compared to the healthy group (***Figure 13A***). On the other hand, in the AF structure the degenerated group has a significant lower value compared to the healthy group (***Figure 13B***). Instead, in the EP structure there is no significant difference between the two groups (***Figure 13C***).

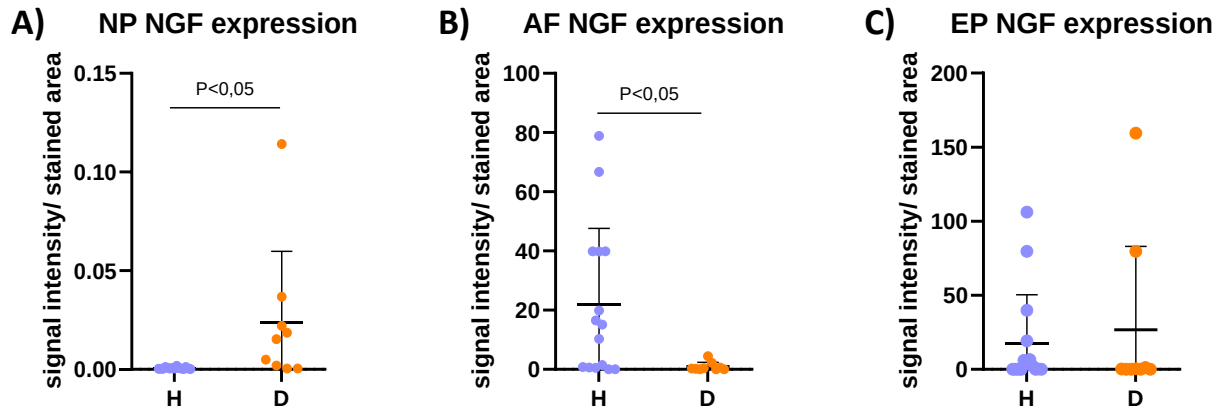


Figure 13*. Difference in NGF signal intensity and stained area ratio between healthy and degenerated IVD group in NP (A, p< 0.05), in AF (B, p< 0.005) and in EP (C, p> 0.5).*

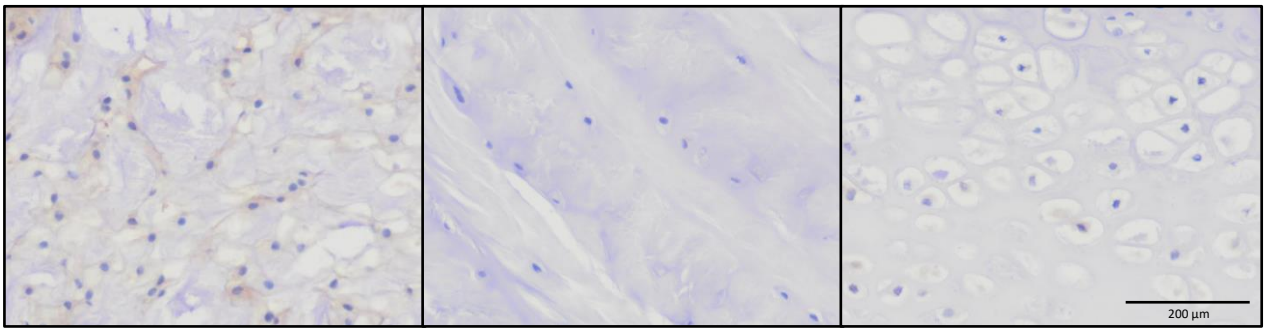


Figure 14. *NGF expression in 5 um healthy rat IVD section (score 0): NP (A), AF (B), EP (C) structures.*

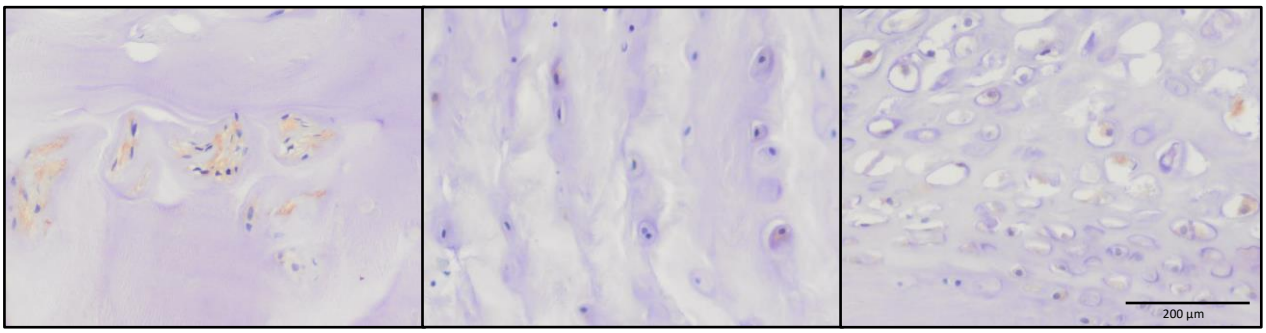


Figure 15. *NGF expression in 5 um degenerated rat IVD section (score 9): NP (A), AF (B), EP (C) structures.*

6.1.3 PGP9.5 expression in rat IVD

In order to evaluate the presence of nerve fibres in the degenerated rat IVD model, the PGP9.5 immunohistochemistry was performed on the sections. In the rat IVD sections, no PGP9.5 expression was detected (***Figure 16***), compared to the rat skin as positive control where the nerve fibres bundles are evidently stained in brown (***Figure 17***). Additionally, positive PGP9.5 nerve bundles were observed outside the IVD (***data not shown***).

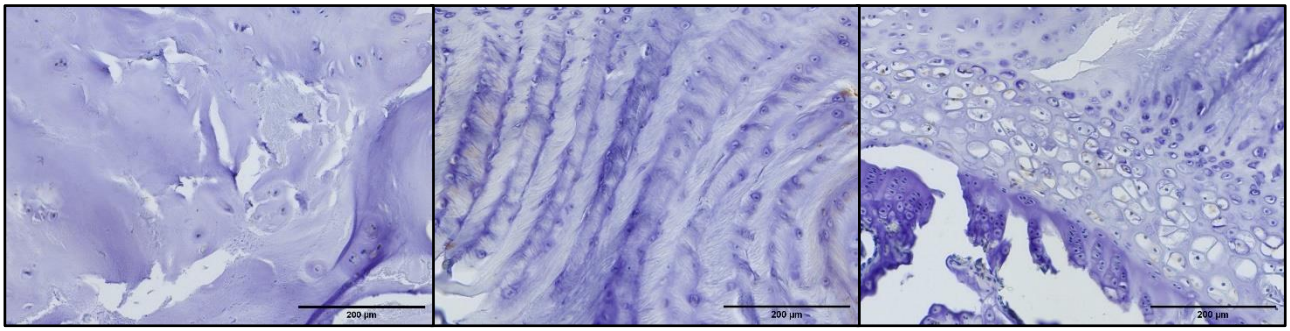


Figure 16. *PGP9.5 expression in 10 um degenerated rat IVD section: NP (A), AF (B) and EP (C) structures.*

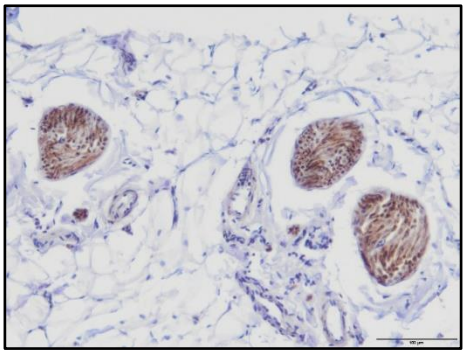


Figure 17*. PGP9.5 IHC positive control: 10 um rat skin section.*

6.2 Celecoxib effect on nerve growth within degenerated canine IVD sections

In order to investigate also the presence of nerve fibres in the degenerated canine IVD model, the PGP9.5 immunohistochemistry was performed on the sections. Similarly to the rat IVD sections, there was no observable PGP9.5 expression in the canine IVD sections (***Figure 18***), compared to the canine skin as positive control, where the nerve fibres bundles are stained in brown, especially around the blood vessels (***Figure 19***).

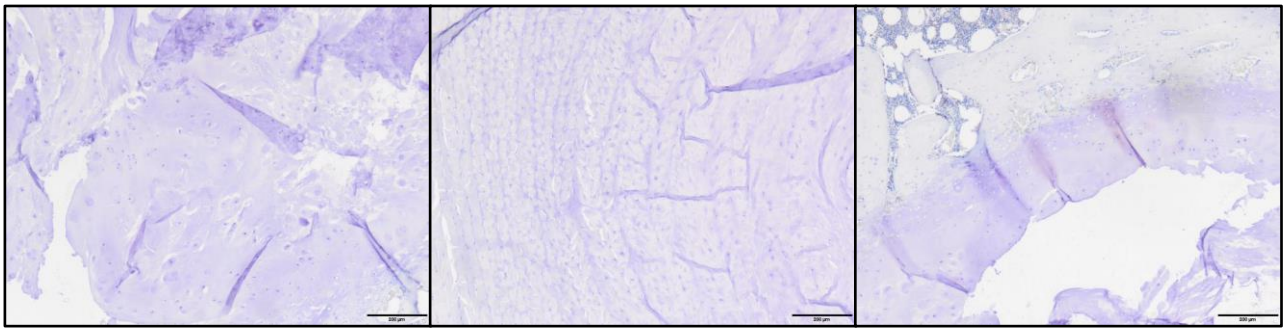


Figure 18. *PGP9.5 expression in 5 um degenerated canine IVD section: NP (A), AF (B) and EP (C) structures.*

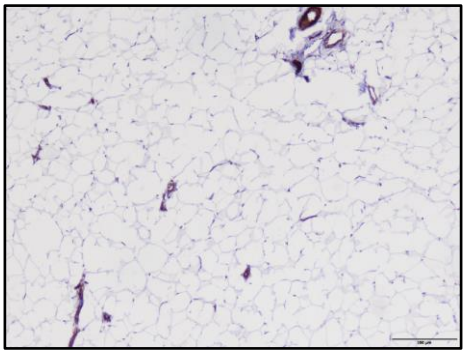


***Figure 19**. PGP9.5 IHC positive control: 5 um canine skin section*

6.3 Pain-related markers expression and celecoxib effect on inflamed human NP and AF cell culture

6.3.1 Effect of TNFα stimulation and CXB treatment on pain-related markers expression in NP cells

To investigate pain marker expression upon a proinflammatory stimulus, NP cell cultures were stimulated with TNFα and treated with CXB. TNFα stimulation was shown to significantly increase the expression of both neurotrophines NGF and BDNF in NP cell culture (***Figure 20A, 20B***). Additionally, CXB in combination with inflammatory stimulus increased NGF and BDNF expression in a dose-dependent manner compared to the untreated TNFα group (***Figure 20***).

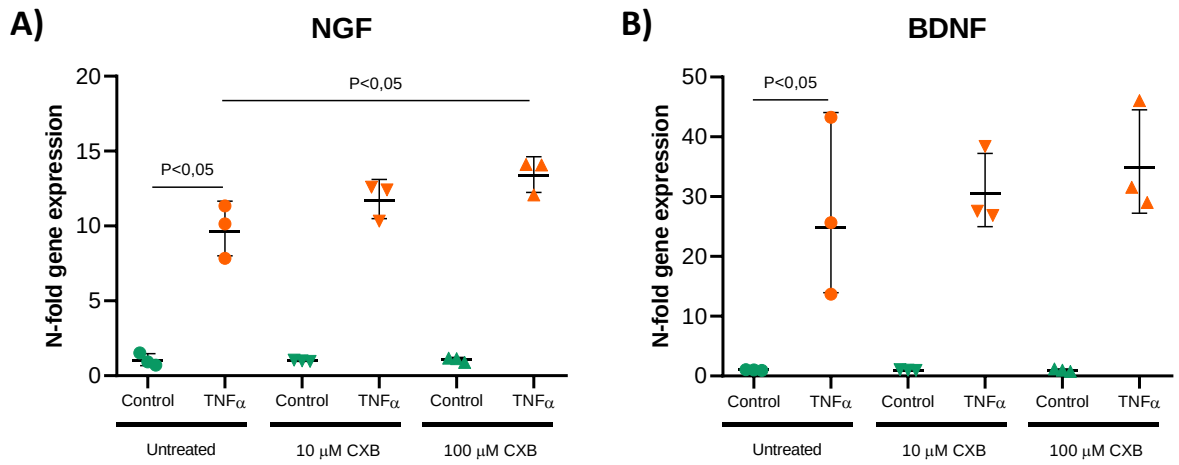


***Figure 20.** Neurotrophines expression (NGF and BDNF) upon 10 ng/mL TNFα stimulation and 10 and 100 uM celecoxib treatment in NP cell culture.*

TNFα had no significant effect on the increase of the neuropeptides CGRP and SP expression in the NP cells (***Figure 21A, 21B***). Additionally, CXB does not have any consistent effect on CGRP and SP expression, in combination with TNFα induction (***Figure 21***).

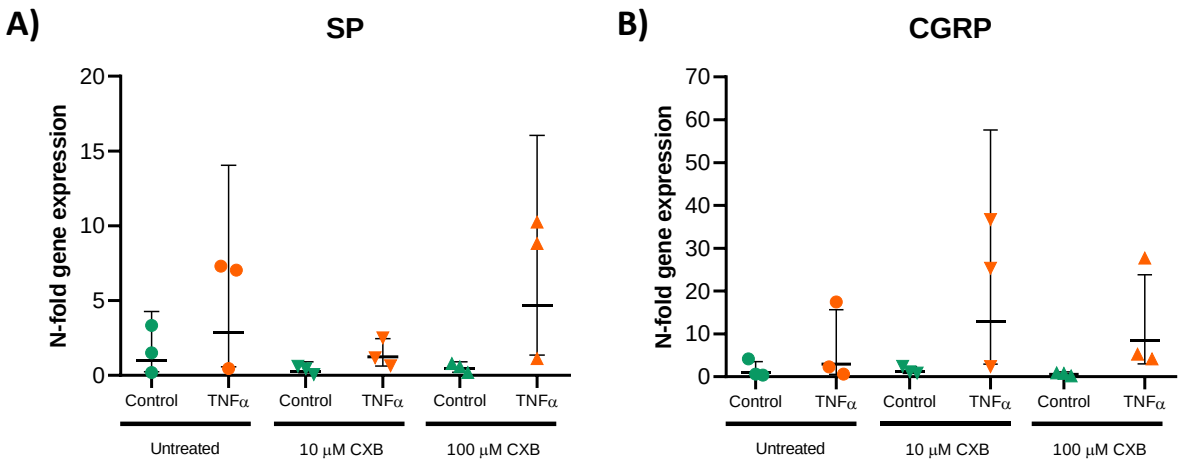
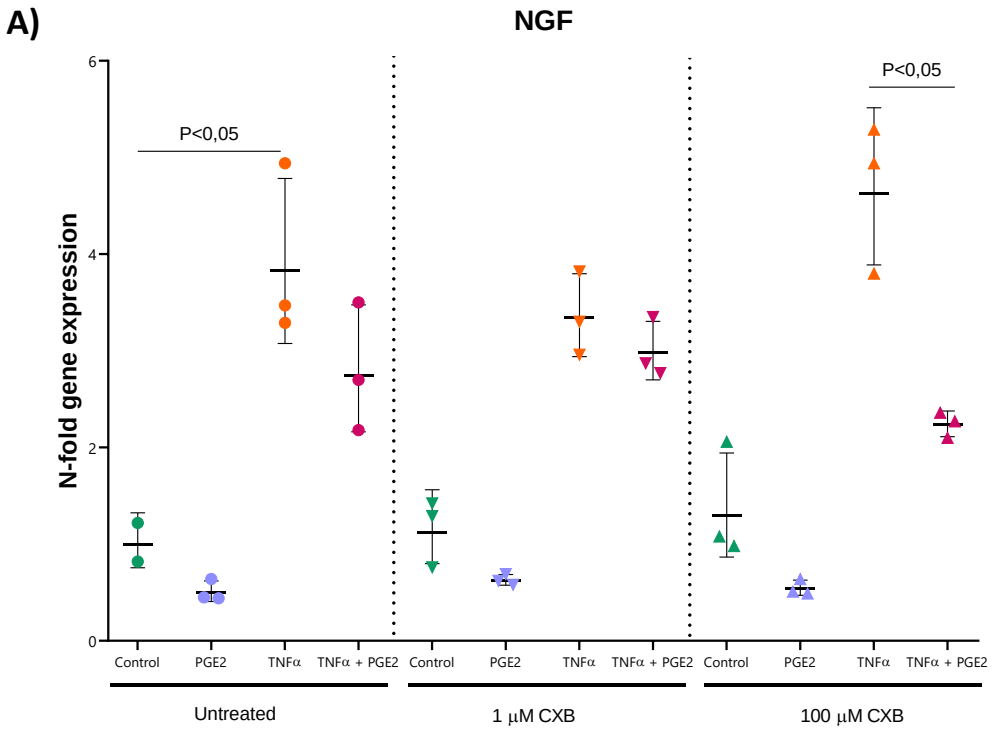


***Figure 21**. Neuropeptides expression (SP and CGRP) upon 10 ng/mL TNFα stimulation and 10 and 100 uM celecoxib treatment in NP cell culture.*

6.3.2 Effect of PGE2 on pain-related markers expression in combination with TNFα stimulation and CXB treatment in NP cells

To investigate the possible restoring property of PGE_2 on CXB effect, exogenous PGE_2 was added to the NP cell culture in combination with the proinflammatory TNF-α and CXB. It was shown that exogenous PGE_2 partially restored NGF expression upon CXB treatment, in particular for the highest CXB concentration (***Figure 22A***). Regarding BDNF, PGE_2 increased its expression further when in combination with TNF-α and CXB or alone (***Figure 22B***).



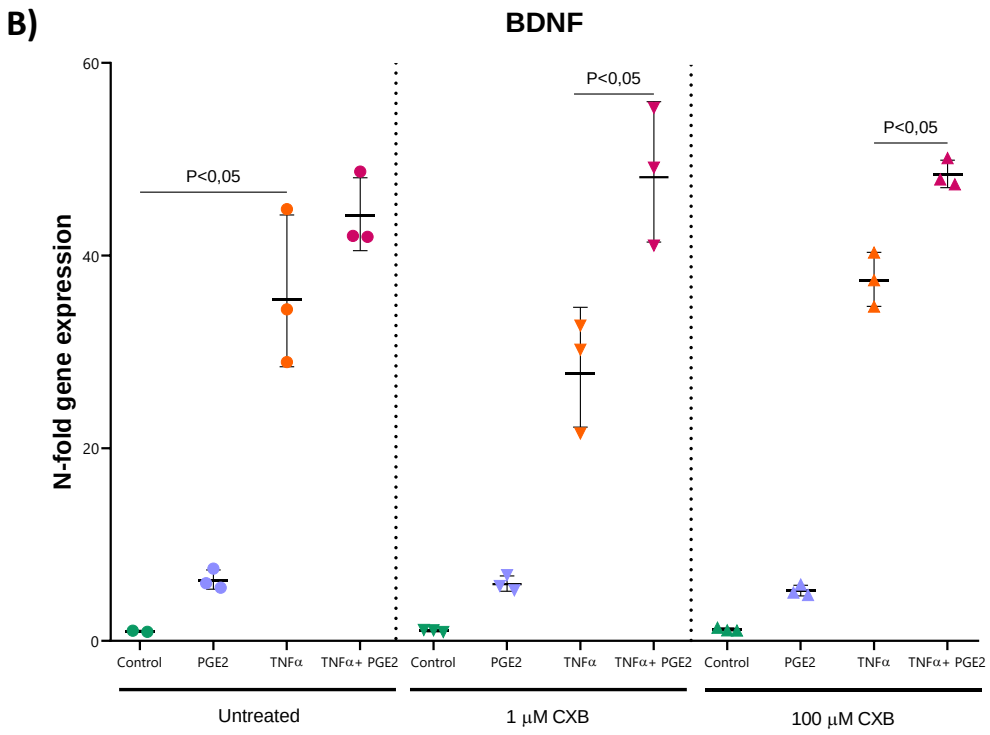
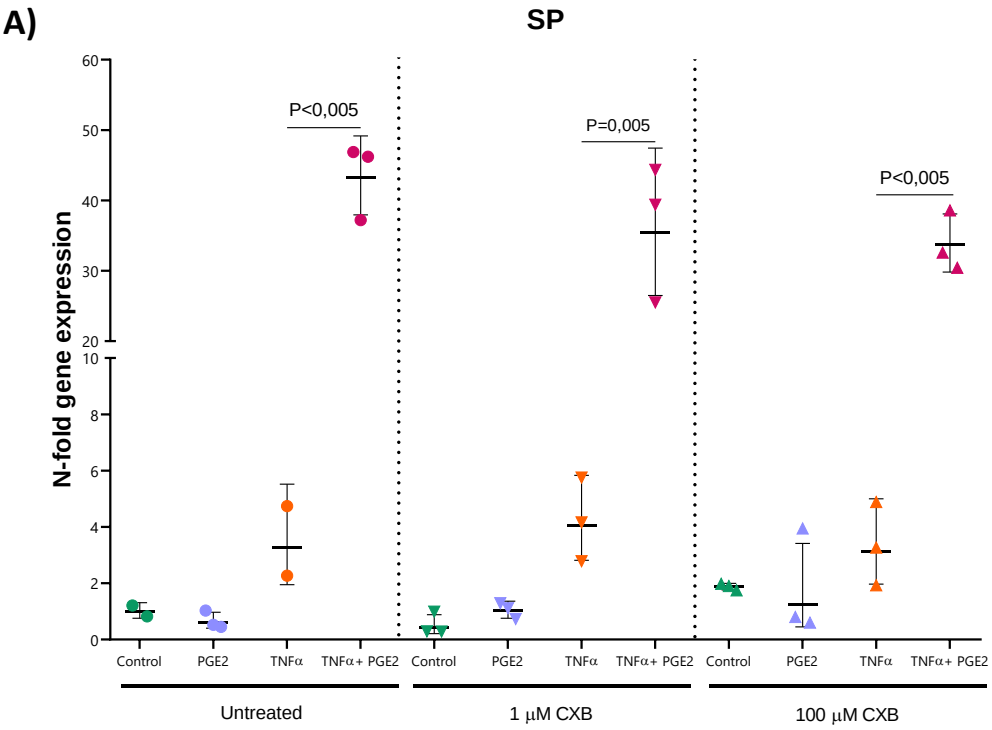


***Figure 22**. Neurotrophines expression (NGF and BDNF) upon 10 ng/mL TNFα stimulation and 1 and 100 uM celecoxib treatment and 10 ng/mL of PGE2 in NP cell culture.*

SP expression seems to follow the same trend as BDNF expression upon PGE_2 treatment, increasing significantly its expression in combination with both CXB concentrations (***Figure 23A***). Additionally, exogenous PGE_2 did not restore CGRP expression upon CXB treatment (***Figure 23B***).



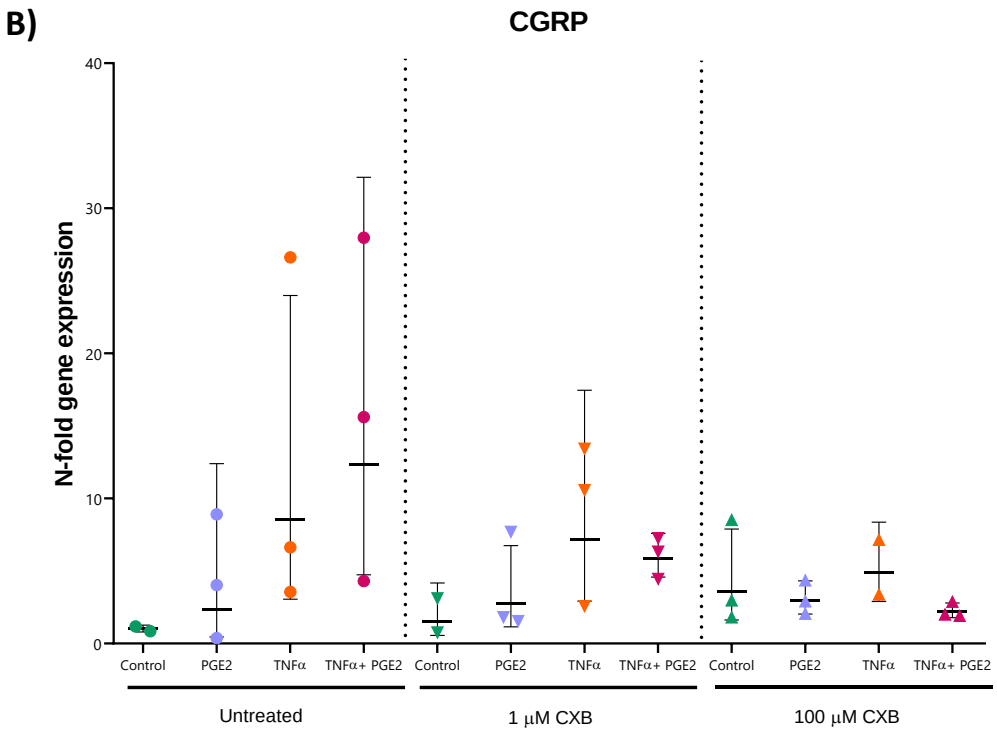
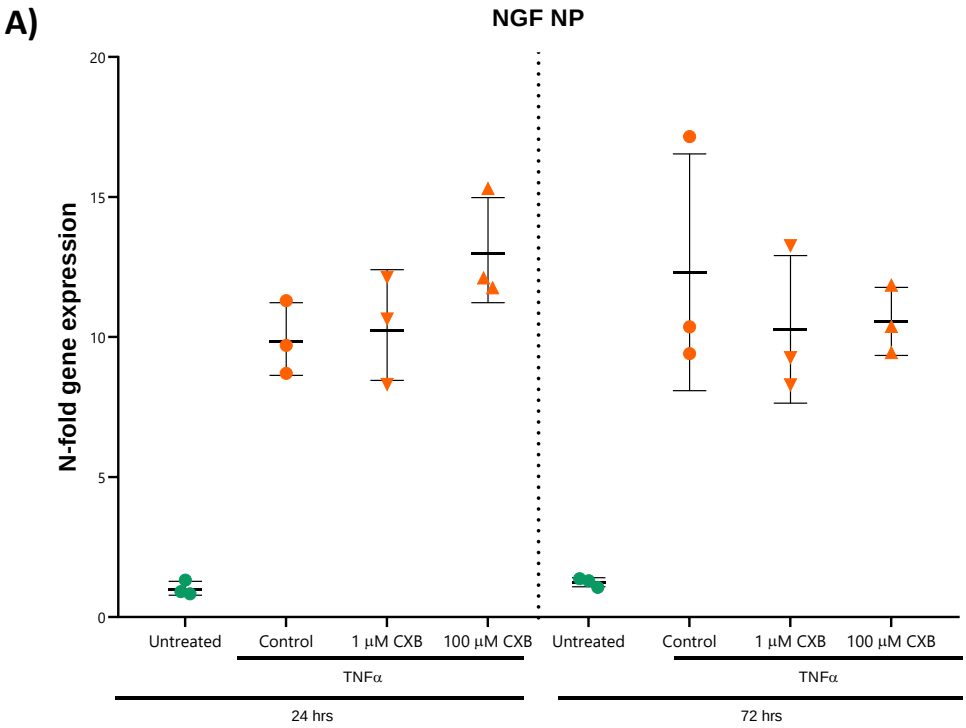


***Figure 23**. Neuropeptides expression (SP and CGRP) upon 10 ng/mL TNFα stimulation and 10 and 100 uM celecoxib treatment and 10 ng/mL of PGE2 in NP cell culture.*

6.3.3 Pain-related markers expression evolution in NP and AF cells

72 hours after treatment, the NGF expression in NP cells is overall constant for all the conditions (***Figure 24A***), but it decreases significantly in AF cells (***Figure 24B***).



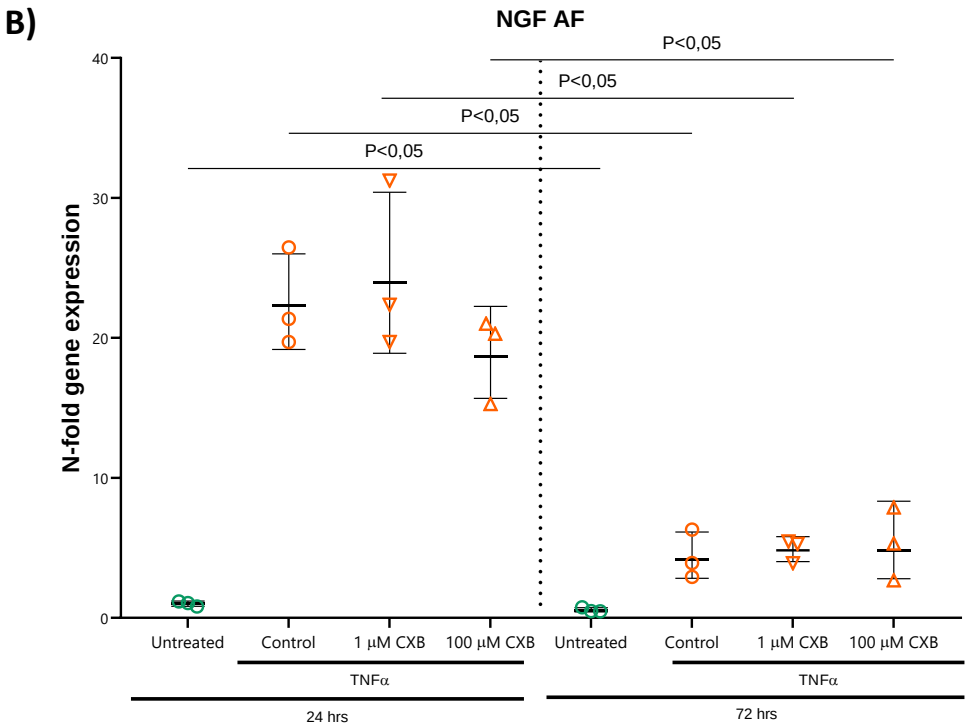
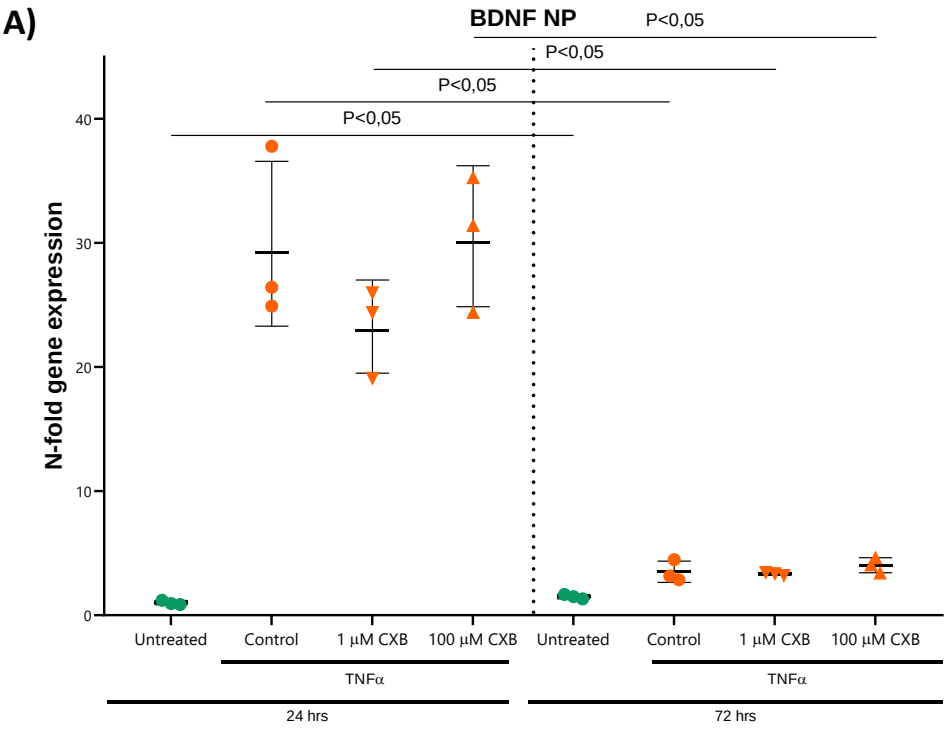


***Figure 24**. NGF expression upon 10 ng/mL TNFα stimulation and 1 and 100 uM celecoxib treatment in NP (B) and AF cells (B), 24 and 72 hours after the stimulation.*

BDNF expression both in NP and AF cells follow a significant decrease after 72 hours after treatment for all the conditions (***Figure 25A, 25B***).



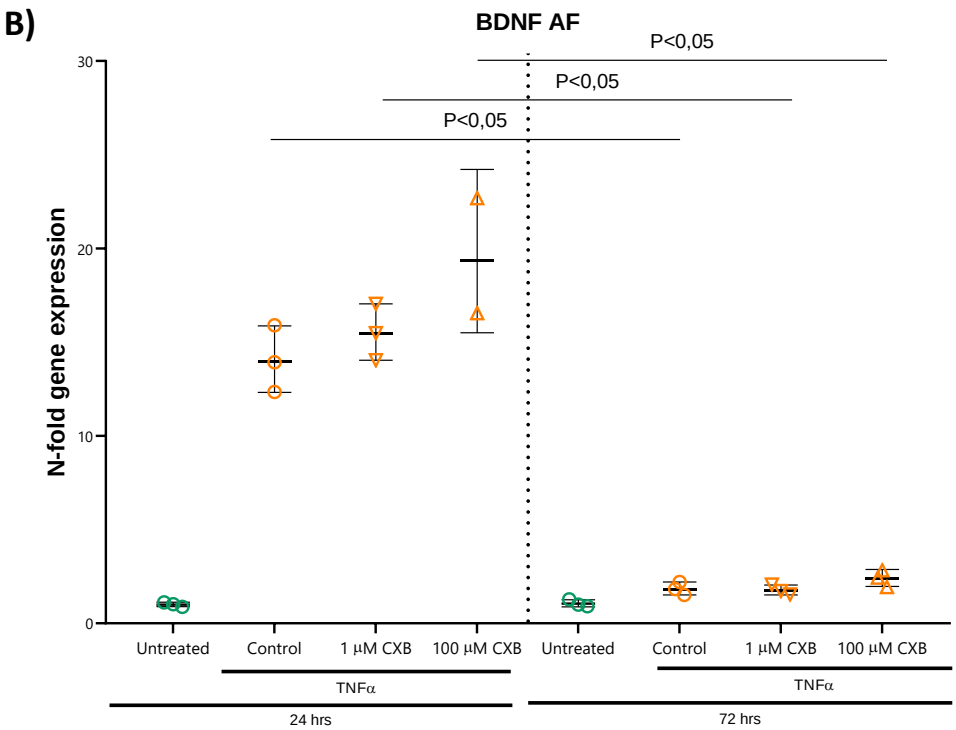


Figure 25. *BDNF expression upon 10 ng/mL TNFα stimulation and 1 and 100 uM celecoxib treatment in NP (A) and AF cells (B), 24 and 72 hours after the stimulation.*

After 72 hours from the treatment, the SP expression was so low that it was not detectable via qPCR (***Data not shown***).

After 72 hours from the treatment, the CGRP expression seemed to significantly increase in NP cells stimulated with TNF-α alone (***Figure 26A***). In contrast, the remaining conditions are constant over time. The same was observed for AF cells (***Figure 26B***).

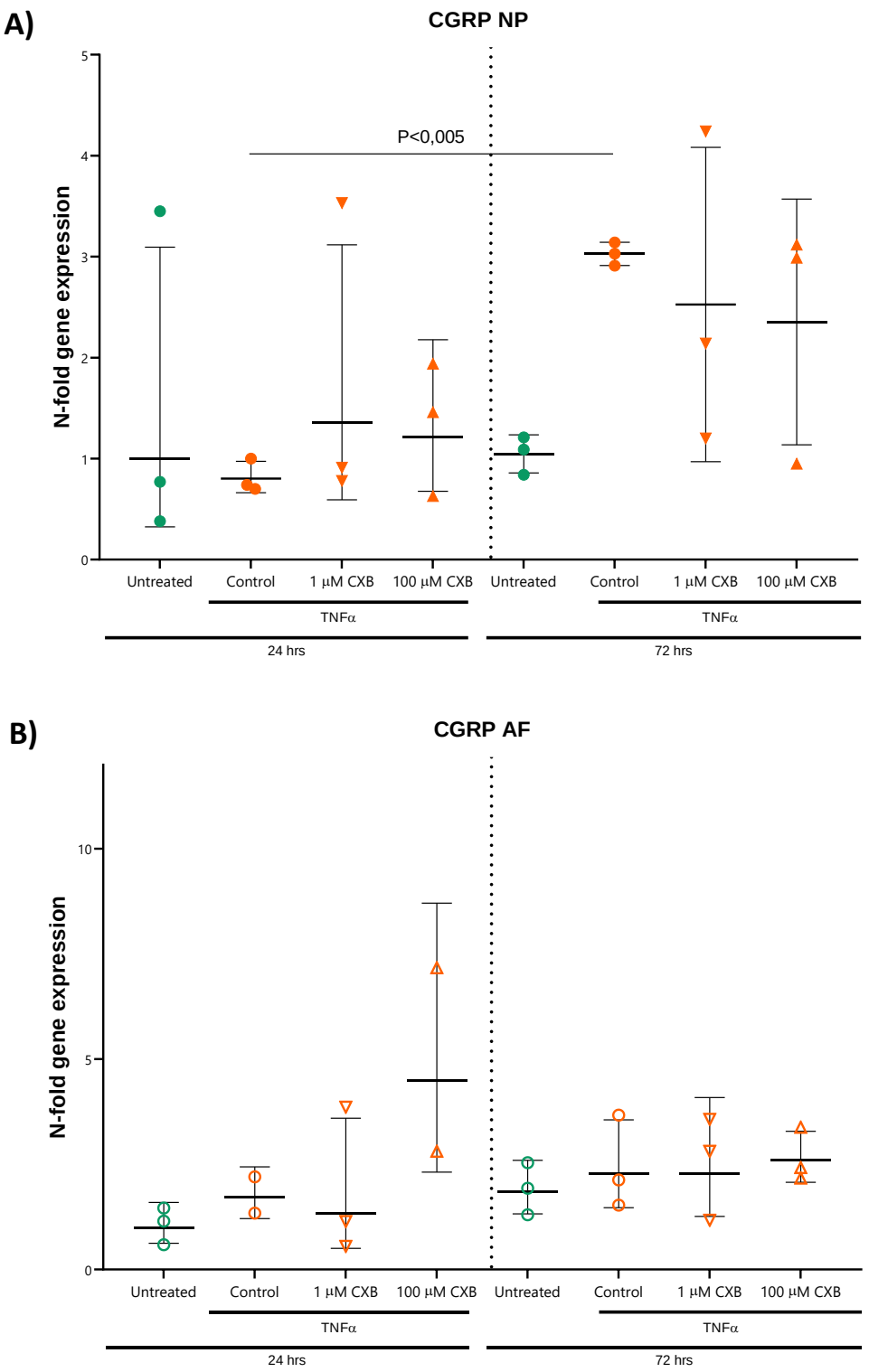


***Figure 26**. CGRP expression upon 10 ng/mL TNFα stimulation and 1 and 100 uM celecoxib treatment in NP (A) and AF cells (B), 24 and 72 hours after the stimulation.*

6.4 Effect of inflammation *and celecoxib treatment* on NGF production and *secretion* by NP and AF cells

The production of NGF by NP and AF cells was analyzed by Western blot. Unfortunately, this evaluation has not given any results, suggesting the protein concentration was bellow detection limit (***Data not shown***).

The secretion of NGF in the culture conditioned media was determined by means of the ELISA. Unfortunately, this evaluation has not showed any signal, suggesting either the absence of the protein in the media or the concentration of NGF too low, under the detectable minimum (31.3 pg/mL) (***Data not shown***).

6.5 Effect of CXB-treated/ non-treated conditioned media on nociceptive fibres outgrowth from mouse dorsal root ganglion (DRG) explants

6.5.1 Cellularity of DRG explants

To investigate the effect of the proinflammatory cytokine TNFα and the possible therapeutical effect of CXB on neurites growth, mouse DRG were explanted and cultured in conditioned media derived from NP or AF cell culture stimulated with TNFα and CXB. First, the number of neurons in each DRG was counted and it was observed that TNFα-conditioned media slightly increased neuron number both for the NP and AF conditioned media (***Figure 28***). Importantly, the conditioned media with the highest CXB concentration seemed to increase significantly the neuron number in combination with inflammatory stimulus, both for NP and AF conditioned media (***Figure 28***).

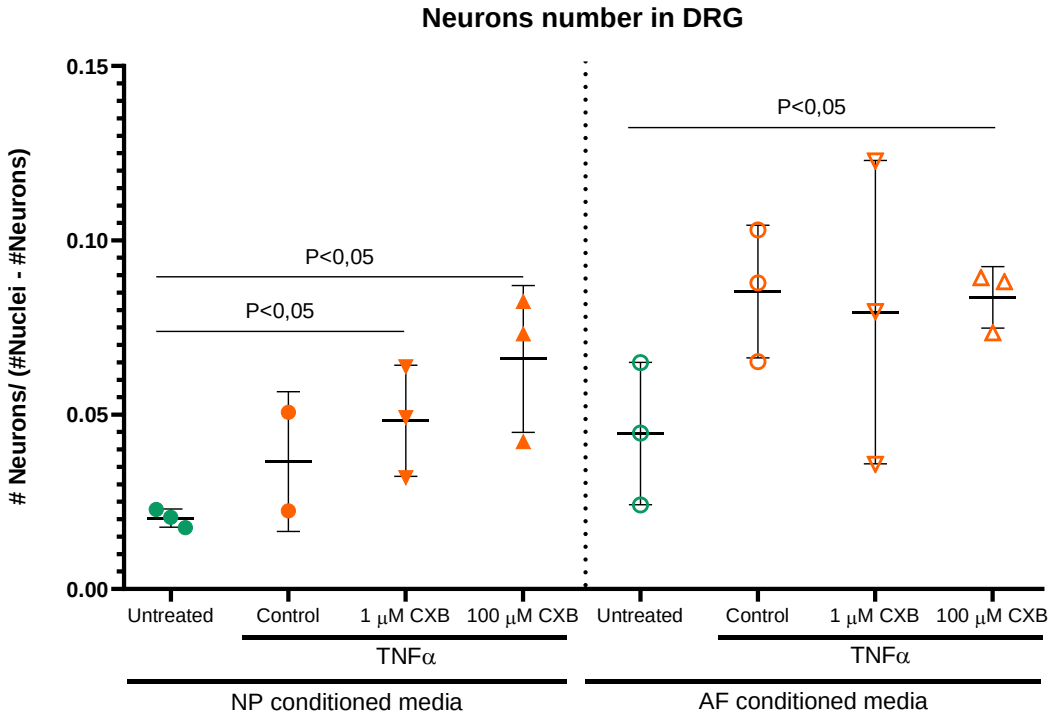


Figure 27*. Average neurons number in the DRG explants, incubated in conditioned media coming from either NP or AF cell culture, stimulated with 10 ng/mL TNFα and treated with 1 and 100 uM celecoxib.*

6.5.2 Neurites outgrowth from DRG explants

Secondly, the average length of the neurites was measured for each DRG, showing that the neurites outgrowth from the mouse DRG explants was not altered by TNFα-conditioned media (***Figure 29***). The neurites outgrowth seems to be constant also upon CXB-conditioned media (***Figure 29***).

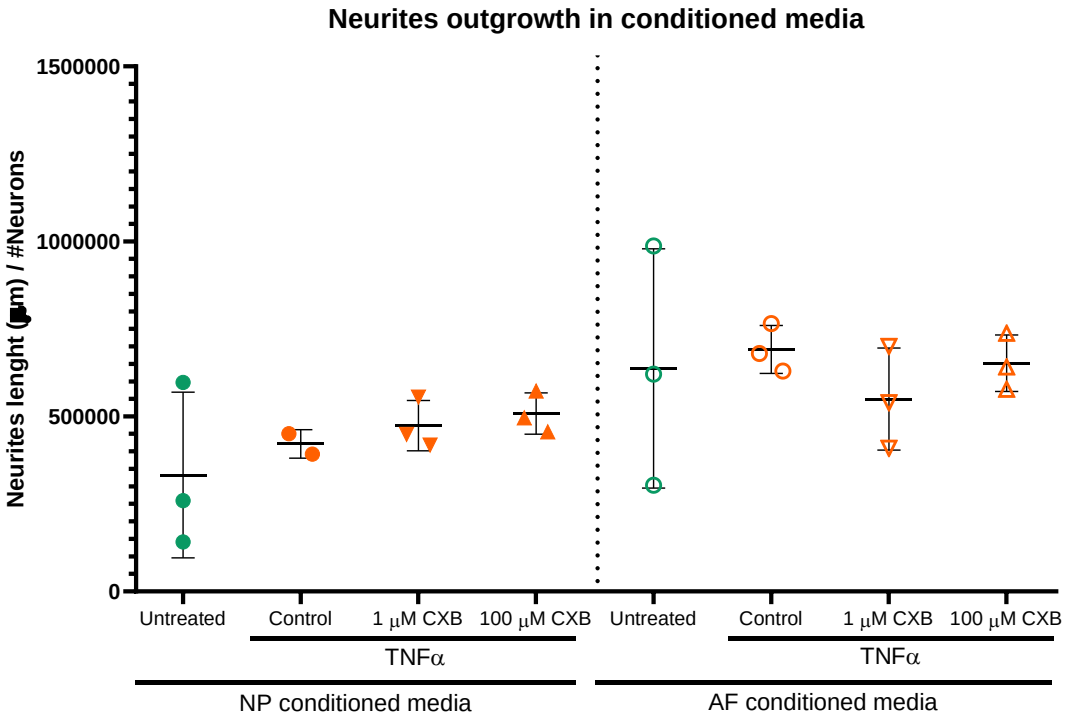


Figure 28*. Average neurites length (µm) in the DRG explants, incubated in conditioned media coming from either NP or AF cell culture, stimulated with 10 ng/mL TNFα and treated with 1 and 100 uM celecoxib.*

7. Discussion and conclusions

The degeneration of the intervertebral disc accounts for 40% cases of chronic low back pain. In CLBP caused by degeneration, pain is mainly caused by the ingrowth of nociceptive nerve fibres into the deeper layer of the IVD. The production of pain-related neuropeptides by IVD cells is also responsible for the triggering of nociceptive stimuli involved in pain perception. Therefore, it is crucial to investigate the nerve ingrowth processes and the expression of pain-related markers in the degenerated IVD to develop new strategies for pain treatment. To address this research question, in this study the morphology and structure of the IVD and presence of nerve markers was evaluated upon the induction of the degeneration in two different animal models

In the rat model, it was shown the degeneration of the IVD decreased the overall cellularity in all of the three major structures of the IVD, each one with a different impact, mainly in the NP and EP. This result is in line with the previous studies (Tellegen et al. 2018) where it was shown IVD cellularity decreased with degeneration.

Indeed, in line with another previous study (Tellegen et al. 2018) that showed that NGF expression is increased in NP and AF structure upon IVD degeneration, here it was also observed increased NGF expression with higher degeneration score, especially in the AF and EP structures. In the NP, the NGF expression was more localized and intense around few cell clusters.

Additionally, PGP9.5 cells were not observed in any of the models. As they were detected in nerve bundles outside the IVD, it is hypothesized that these cells might need longer times to grow into the IVD. In fact, the degeneration of the IVD in humans is a process that takes decades before it exacerbates into lower back pain caused by the ingrowth of nociceptive fibres into the degenerated IVD. Contrastingly, in the present study, the post IVD degeneration induction time elapsed before the termination was 8 weeks for the rat model and 16 weeks for the canine model. That is the reason why it is very reasonable to think that it could be necessary more time between the induction of the degeneration of the IVD in the animal models and their termination, in order to see the growth of the neurites into the degenerated IVD, as it happens in human.

Subsequently, we evaluated the effect of TNFα stimulation on expression of pain markers in human NP and AF cells. TNFα was shown to play an overall stimulatory effect on pain-related marker expression from NP cells, such as the neurotrophines NGF and BDNF, which is in line with findings from a previous study (Binch et al. 2014). This effect, however, was not so evident in the case of neuropeptides, such as CGRP and SP, which is in contrast with the previous study (Binch et al. 2014).

Moreover, this study tried to cast light on the therapeutical effect of celecoxib on degenerated IVD. In fact, an in vivo study (Tellegen et al. 2018) showed how CXB reduced the overall degeneration grade of the IVD and NGF expression, which is known to play a role as neurotrophin and sensitizer of the nociceptive fibres. The result of this study is drastically in contrast with the previous one, since CXB did not decrease the expression of pain markers and neurotrophines . On the contrary, it significantly increased the expression of NGF and BDNF in NP cell culture in a dose-dependent manner. However, this was not true for CGRP and SP expression in NP cell culture. Therefore, these results suggest the presence of other molecular player and the different environment in vivo, represented by the surrounding tissues and the presence of other cell types that could have an impact on the therapeutical effect of CXB on pain-marker expression.

In a previous study (Alimasi et al 2013), it has been shown in human NP that NGF expression is increase upon the treatment with another COX2-selective inhibitor, NS-398, in a dose-dependent manner similar to CXB. In this study, it was proven that the addition of exogenous PGE_2 was effective in restoring NGF expression. A negative feedback mechanism was hypothesized . In detail, when the PGE2 production by the enzyme COX2 is inhibited by a COX2-selective inhibitor, the negative feedback played by PGE2 on NGF expression is disrupted, leading to an increase of NGF expression. Indeed, in this way it is also explained the dose-dependent manner observed in this study, the higher is the celecoxib concentration the lower is the PGE2 production and ultimately the weaker the inhibition on NGF expression. In fact, also in the present study, the addition of exogenous PGE_2 to the NP cells stimulated with TNFα and treated with CXB at different concentrations seems to be significantly effective in reducing NGF expression, especially for the highest CXB concentration. In contrast, exogenous PGE_2 further increased the expression of BDNF and SP upon TNFα stimulation and CXB treatment, meaning these markers are independent of mechanisms involving PGE_2 feedback

Furthermore, this study wanted to investigate the effects of the neurotrophines secreted by inflamed NP and AF on the neurite growth responsible for the nociception of the degenerated IVD in vivo. To do so, mouse DRG explants were cultured in conditioned media derived from NP and AF cell culture. Additionally, the effect of CXB on neurotrophin expression and therefore on neurites growth from the DRG explants was also evaluated. A previous report (Krock et al. 2014) had a similar setup using instead a rat adrenal pheochromocytoma (PC12) cell line, which expresses the receptor for NGF. When exposed to NGF they take on a neuronal-like phenotype and they are commonly used to study neuronal differentiation and neurite sprouting. Upon culturing in degenerated IVD

conditioned medium or healthy IVD, neurite outgrowth was measured. It was shown that degenerated IVD media induced neurite sprouting in a significantly greater percentage of cells compared to cells cultured in healthy IVD media indicating that painful, degenerating IVDs produce factors that promote neurite growth (Krock et al. 2014). The same research question was addressed in the present study, by measuring first the number of neurons and second the average neurite length. The number of neurons in the DRGs are slightly higher for both conditioned media coming from NP and AF culture stimulated with TNFα compared to the control. Indeed, in line with the previous results of this study, neuron numbers were also slightly higher for both NP and AF conditioned media stimulated with TNFα and treated with CXB. We hypothesize that neuron death is inhibited by pain markers released by inflamed IVD cells. Therefore, even if, the gene transcription of the pain markers is overall reduced after 72 hours after treatment, they are presumably still accumulated in the media at enough concentration in order to influence the neurons number of the DRGs. However, neurites outgrowth from DRG explants seem not to be affected neither by conditioned media coming from both NP and AF culture stimulated with TNFα with or without CXB treatment. A possible reason for these results is the long DRGs explant culture, resulting in the confluency of the explants and therefore the impossibility to see the effect of pain markers present in the conditioned media on neurites outgrowth.

To conclude, in order to study nerve growth into degenerated IVD, it might be necessary to set up proper models which resemble the physiological mechanism and process of IVD degeneration in human, besides providing enough time for nerve fibres to investigate the innervation of the degenerated IVD. Moreover, it is also important to evaluate the possibility that low back pain might also derive from other nerves outside the disc, besides IVD innervation, however this hypothesis requires further investigation. In addition, regarding the in vitro work in the present study, it has been shown that CXB promotes NGF expression as opposed to in vivo studies. Therefore it is fundamental to keep in mind that cell models must be interpreted carefully when it comes to modelling the process of the IVD degeneration which is a condition where several components and the environment play a crucial role in its exacerbation.

In conclusion, it is clear how further studies are necessary to investigate more in detail the mechanisms underlying the inflammation and the pain perception, represented by neurotrophines and neuropeptides expression and nociceptive fibres ingrowth within the degenerated IVD and the therapeutical effect of CXB in vivo.

8. Limitations and improvements

This study has some limitations and hereafter some improvements are suggested.

The first limiting aspect of this study is the absence of at least three biological replicates, besides the three technical ones, of the cultured NP and AF cells, since they are derived from just one donor, in order to increase the biological variability and the statistical significance of the results.

Additionally, during the optimization of the immunohistochemistry for the NGF and PGP9.5 of the rat and canine IVD sections, it was very problematic the attaching properties of the samples sections on the glasses upon the antigen retrieval step. To improve this, it could be useful maybe to reduce the thickness of the sections, add an extra step of extra fixation and attachment to the glass or reduce the antigen retrieval temperature, prolonging this step for longer time.

As mentioned before, another aspect that could be improved in this study is the limited time between the surgery of the rat and canine models and study termination, probably responsible for the absence of PGP9.5 positive nerve fibres in the degenerate IVD.

Regarding the DRG experiments, it has also not been taken into consideration the specie-specific interactions between the human IVD cells culture and the mouse DRG explants. It is likely the mouse DRG explant responds in a different way to the pain markers produced by human IVD cells, compared to the human ganglions. In fact, both the pain markers and their receptors do not share a 100% similarity between human and mouse species (Paoletti et al. 2015). This difference could be crucial in explaining possible differences and dynamics of the neurites growth from mouse DRG explants. To improve this aspect, it would be interesting to take in consideration a human neural cell line, such as NT2 (Darbinian, 2021) to resemble more the species-specific interactions present in vivo.

Indeed, in order to see a greater effect of pain markers produced by IVD cell culture on the DRG explants, it would be recommendable to increase the concentration of the pain markers in the medium, by shortening the period after stimulation, therefore avoiding the expression decrease of some released factors, such as NGF (Alimasi et al. 2013), and also by shortening the incubation of DRGs in the conditioned media (<48 hrs), in order to avoid the confluency of the neurons.

Another limitation encountered during this study is the very small amount of SP and CGRP mRNA extracted from NP and AF cells, leading to high variability of the qPCR results. In fact, for this reason it was difficult to do constant measurements of the gene expression of these two peptides. Most

probably to solve this aspect it might be enough to increase the number of plated cells, in order to increase the amount of mRNA that could be extracted, reducing the variability among the measurements.

To conclude, in this project it has been mainly investigated the mRNA expression profile of the pain-related markers in the IVD cell cultures via qPCR, but it would be interesting also to investigate the protein expression profile for those pain markers via ELISA or Western blot, both on cell lysate and media, in order to also study whether protein levels correspond to the effect observed on gene expression

9. Future studies

From the results of the present study, it could be possible to think about some follow ups to further investigate the mechanisms and processes involved in the degeneration of the IVD and the consequent expression of pain markers responsible for the pain perception.

First of all, it would be interesting to investigate the expression of pain-related markers also in EP cells besides AF and NP ones, since the endplate is one of the first structure of the IVD to be involved by the degeneration, degrading its matrix, creating tears and fissures which are the main routes for nociceptive nerve fibres to grow into the inner layer of the degenerated IVD.

Regarding the neurites growth model composed by mouse DRG and conditioned media derived from IVD cells, it would be interesting to selectively inhibit specific pain marker activity by means of antibodies or siRNA technique to investigate their specific effect on neurites outgrowth.

To conclude, a co-culture model composed by human NP and/or AF cells or tissue and human DRG explants embedded in collagen type II scaffold would be a valid and innovative tool which could resemble the physiological IVD environment, in order to investigate better the interaction between the IVD components and the nervous system responsible for pain perception.

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