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The Effects of ATP and Adenosine on the Extracellular Matrix Biosynthesis Via Purinergic Pathways

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THE EFFECTS OF ATP AND ADENOSINE ON THE EXTRACELLULAR
MATRIX BIOSYNTHESIS VIA PURINERGIC PATHWAYS

By

Silvia Daniela Gonzales

A DISSERTATION

Submitted to the Faculty
of the University of Miami
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THE EFFECTS OF ATP AND ADENOSINE ON THE EXTRACELLULAR
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Silvia Daniela Gonzales

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The Effects of ATP and Adenosine on the Extracellular (August 2014)
Matrix Biosynthesis Via Purinergic Pathways

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Chun-Yuh Charles Huang

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Low back pain causes distress and suffering to patients. The impact of low back pain creates a major socio-economic burden in industrialized societies as well. As the leading cause of disability, low back pain affects more than 80% of the US population at some point in life. Intervertebral disc (IVD) degeneration has been closely associated with low back pain, stimulating interest in finding the causes that lead to IVD degeneration. Therefore, understanding the mechanisms involved in the maintenance of IVD composition may shed light to development of novel therapies for IVD degeneration and low back pain.

IVD cells are responsible for maintaining adequate rates of biosynthesis, breakdown and accumulation of extracellular matrix (ECM) constituents in order to preserve the quality and integrity of the ECM and consequently, the IVD's functional properties. In order to do that, IVD cells produce energy in the form of adenosine triphosphate (ATP). While mechanical stimulation has shown to promote ECM biosynthesis and ATP release, the effects of direct stimulation of the purinergic pathway by extracellular ATP and its derivative, adenosine, on the ECM biosynthesis in IVD cells have not been elucidated. Therefore, the objective of this dissertation is to study the effects of extracellular ATP and

adenosine on the ECM biosynthesis of IVD cells, in order to better understand the role of the purinergic pathway in the maintenance of IVD composition.

A noninvasive system for detecting membrane potential variations induced by ATP stimulation using a potentiometric dye and image processing techniques was developed. The results indicate that IVD cells have different responses to exogenous ATP stimulation in monolayer and 3-dimensional cultures. Furthermore, AF and NP cells exhibited distinct patterns and magnitudes of membrane potential changes. The ATP-induced response was found to be the result of activation of P2 purinergic receptors in IVD cells.

The effects of extracellular ATP on the ECM biosynthesis of IVD cells were studied. Results show up-regulation of aggrecan and type II collagen gene expressions in NP and AF cells by extracellular ATP. Inhibition of gene expression by ATP receptors antagonist suggests that ATP receptors are involved in the ECM biosynthesis in IVD cells. In addition, NP cells were found to be less sensitive to low concentrations of extracellular ATP than AF cells while NP cells exhibited higher accumulation of PG, collagen, and intracellular ATP compared to AF cells.

Extracellular adenosine treatment promoted ECM biosynthesis and intracellular ATP production in IVD cells. Gene expression of aggrecan and type II collagen in IVD cells was also up-regulated by extracellular adenosine. Moreover, inhibition of gene expression by adenosine receptors antagonists suggests that adenosine receptors are involved in biological activities in the IVD. The results also suggest that hydrolysis of extracellular ATP promoted by mechanical loading regulates ECM biosynthesis of IVD cells via adenosine formation.

The findings of this dissertation contribute to further understanding the role of ATP and adenosine on the ECM biosynthesis of IVD cells. Given that ATP production is fundamental for ECM biosynthesis, this study can provide insight into the role of the purinergic pathway on the ECM biosynthesis in the IVD.

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CHAPTER 1: INTRODUCTION AND SPECIFIC AIMS

1.1 INTRODUCTORY REMARKS

Low back pain causes distress and suffering to patients and is a major socio-economic burden in industrialized societies. As the leading cause of disability, low back pain affects more than 80% of the US population at some point in life (How-Ran, et al., 1999). Intervertebral disc (IVD) degeneration has been closely associated with low back pain, stimulating interest in finding the causes that lead to IVD degeneration. Anatomical and cellular changes are normal age-related modifications in disc structure. Nonetheless, it is difficult to differentiate age-related changes from pathological conditions that cause IVD degeneration (Lotz, et al., 2002).

The mechanical properties of the spine are defined by its functional units, called motion segments, which consist of two adjacent vertebrae, an IVD, ligaments and apophysial joints. The role of the ligaments, the facet joints and the IVD is to provide stability to the functional unit of the spine. Since the role of each of the three stabilizing elements is complementary to the role of the other two elements, damage to any of them can compromise the stability of the motion segment (Sharma, et al., 1995). The IVD is an avascular structure that contributes to the mechanical properties that facilitate flexion, bending and torsion of the spine and transmission of loads through the spinal column. Changes in IVD morphology caused by aging or/and disc degeneration compromise the mechanical properties of healthy discs.(Roughley, 1976).

1

The biomechanical properties of the IVD are maintained by the composition and organization of its extracellular matrix (ECM). The ECM is a dynamic structure which undergoes constant remodeling by proteinases and aggrecanases (Tsang, et al., 2010). IVD cells are responsible for maintaining the proper homeostatic balance of biosynthesis, breakdown and accumulation of ECM constituents (Ohshima, et al., 1995). These cellular processes determine the quality and integrity of the ECM and thus, the disc's mechanical response (Buschmann, et al., 1995).

Adenosine-5'-triphosphate (ATP) is the major form of energy in the cell and is a powerful signaling molecule that mediates regulatory roles in short and long-term processes via purinergic pathways (Edwards, 1994). The IVD cells consume glucose and oxygen to produce energy in the form of ATP, which is fundamental for the high energy demanding process of ECM synthesis. Due to the avascular nature of the disc, the nutrient supply to produce ATP is scarce, especially for cells in the center of the IVD. Mechanical loading has showed to influence ECM synthesis. Since dynamic loading promotes ATP production and release from IVD cells (Czamanski, et al., 2011, Fernando, et al., 2011), extracellular ATP may activate the underlying mechanotransduction pathways that affect ECM synthesis. Moreover, high accumulation of extracellular ATP found in the NP (Wang, et al., 2013) may play an important role in maintaining a healthy ECM structure of the IVD. Therefore, it may be possible to stimulate the same cellular response (e.g. ECM biosynthesis) via mechanotransduction pathways by extracellular ATP without applying external forces. In addition, mechanical loading has also demonstrated to decrease extracellular ATP content in the NP (Wang, et al., 2013), suggesting that compressive

loading promotes hydrolysis of ATP. Extracellular ATP is catabolized into adenosine by a cascade of ectonucleotidases (Haskó and Cronstein, 2004, Zimmermann, 2000). Thus, since adenosine can also mediate a variety of cellular functions, high accumulation of extracellular ATP and adenosine due to the disc's avascular nature may be involved in the regulation of tissue functions, including ECM biosynthesis. Therefore, the main objective of this study was to investigate the effects that direct stimulation of the purinergic receptor pathway by extracellular ATP and adenosine on mediating the ECM synthesis of IVD cells.

1.2 SPECIFIC AIMS

The following are the hypotheses of this study:

1. Exogenous ATP application to the culture medium elicits a membrane potential change in IVD cells via purinergic pathways.

2. ATP upregulates aggrecan and type II collagen gene expression via ATP receptors, promotes the deposition of extracellular matrix constituents and increases intracellular ATP content in IVD cells.

3. Adenosine upregulates aggrecan and type II collagen gene expression via adenosine receptors, promotes the deposition of extracellular matrix constituents and increases intracellular ATP content in IVD cells.

To test these hypotheses, the following are the specific aims of this project.

Specific Aim 1: To develop a non-invasive measuring system of ATP-induced membrane potential change of IVD cells.

The fluorescent dye di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate (di-8-ANEPPS) was used to investigate the response of IVD cells to ATP by examining the relative change in membrane potential using digital imaging processing tools. To determine if ATP receptors are activated in IVD cells, the cells were treated with an antagonist of ATP. Gene expression of ATP receptors was examined to confirm the transduction via purinergic pathways.

Specific Aim 2: To determine the relative gene expression change of aggrecan and type II collagen, the amount of proteoglycans and collagen deposition and intracellular ATP content in IVD cells treated with ATP.

IVD cells seeded in agarose were cultured with 100 μM of ATP for 16 hours and the gene expression change of aggrecan and type II collagen was determined using real-time PCR. Relative changes in gene expression were quantified using the $2^{-\Delta\Delta C_T}$ method followed by a statistical analysis to determine significant differences in gene expression. In addition, IVD cells were treated with an ATP receptors antagonist to examine if ATP receptors are involved in the gene expression of the anabolic genes aggrecan and type II collagen. To investigate the effects of ATP on the ECM biosynthesis, IVD cells seeded in agarose were cultured under different concentrations of ATP for 21 days. The dimethylmethylene blue assay (DMMB) for sulphated glycosaminoglycans was used to measure the proteoglycan content and the hydroxyproline wear analysis was used to

measure the collagen content at the end of the experiment. In addition, IVD cells seeded in agarose were treated with 100 μM of ATP for 2 hours and the intracellular ATP content was determined. Statistical analysis tools were used to determine significant differences between the Control and treatment groups.

Specific Aim 3: To determine the relative gene expression change of aggrecan and type II collagen, the amount of proteoglycans and collagen deposition and intracellular ATP content in IVD cells treated with adenosine.

IVD cells seeded in agarose were cultured with 100 μM of adenosine for 16 hours and the gene expression change of aggrecan and type II collagen were determined using real-time PCR. Relative changes in gene expression were quantified using the $2^{-\Delta\Delta C_T}$ method followed by a statistical analysis to determine significant differences in gene expression. In addition, IVD cells were treated with adenosine receptors antagonists to examine if adenosine receptors are involved in the gene expression of the anabolic genes aggrecan and type II collagen. To investigate the effects of adenosine on the ECM biosynthesis, IVD cells seeded in agarose were cultured under different concentrations of adenosine for 21 days. The DMMB assay and the hydroxyproline wear analysis were performed at the end of the experiment. In addition, IVD cells seeded in agarose were treated with 100 μM of adenosine for 2 hours and the intracellular ATP content was determined. Statistical analysis tools were used to determine significant differences between the control and treatment groups.

1.3 CONTENTS OF THIS DISSERTATION

The overall objective of this dissertation was to investigate whether direct stimulation of the purinergic receptor pathway by extracellular ATP and adenosine affects the extracellular matrix biosynthesis of IVD cells, in order to better understand the mechanisms involved in the maintenance of IVD composition and its relation with low back pain. A background of the intervertebral disc, extracellular matrix components, ATP and adenosine is given in Chapter 2. To achieve the objectives of this study, a noninvasive system for detecting membrane potential variations induced by ATP stimulation was developed. Experiments were carried out to investigate the effects of ATP and adenosine on the ECM biosynthesis of IVD cells.

In Chapter 3, a non-invasive system to measure transient ATP-induced membrane potential changes is described. The system uses the potentiometric dye di-8-ANEPPS and digital imaging processing techniques to record membrane potential changes of IVD cells in both monolayer and 3-dimensional cultures.

In Chapter 4, relative changes in aggrecan and type II collagen gene expressions, proteoglycan and collagen depositions and intracellular ATP production measurements of IVD cells in 3-dimensional culture treated with ATP are presented. A mechanobiological pathway for regulating ECM biosynthesis via ATP metabolism is proposed.

In Chapter 5, measurements of relative changes in aggrecan and type II collagen gene expressions, proteoglycan and collagen depositions and intracellular ATP production of IVD cells in 3-dimensional culture treated with adenosine are described.

Gene expression of adenosine receptors are shown. Chapter 6 summarizes the major findings of this study. Recommendations for future work are also presented.

CHAPTER 2: BACKGROUND AND SIGNIFICANCE

The IVD is a fibrocartilagenous structure that lies between the vertebral bodies and has unique architectural, cellular and mechanical properties. It is composed of an outer layer annulus fibrosus (AF), a central nucleus pulposus (NP) and two hyaline cartilage endplates (CEP) (Figure 1A). The AF is made up of 10-12 sheets called lamellae with fibers running parallel within each lamella and oriented at approximately 60° to the vertical axis (Figure 1B)(Urban, 2003). The AF is a fibrous structure rich in collagen that allows bending and torsion of the disc. As the main load bearing element of the IVD, the alternating alignment of fibers within the AF allow the tissue to withstand applied forces (Lundon and Bolton, 2001). Type I collagen, proteoglycans, elastin and chondrocyte-like cells constitute the main elements in the AF region (Lundon and Bolton, 2001). The NP is formed by a highly hydrated aggrecan-containing gel in which collagen fibers are embedded randomly and the elastin fibers are organized radially. Located in the center of the IVD, the jelly-like NP resists compression by providing a high swelling pressure and maintaining hydration under large external loads (Roughley, 1976). The composition of the NP allows it to redistribute forces in all directions of the AF and CEP (Lundon and Bolton, 2001). The cells present in the IVD are responsible for maintaining a matrix consisting of proteoglycans, collagens, other noncollagenous proteins and elastin (Lundon and Bolton, 2001). The CEP is a thin layer of hyaline cartilage of less than 1 mm in between the IVD and the vertebral bodies. This structure, which is the anatomic boundary of the IVD, prevents the NP from leaking out and provides lateral support to the IVD and vertebral bodies during load transmission. Figure 2 shows the transverse view of a porcine lumbar disc.
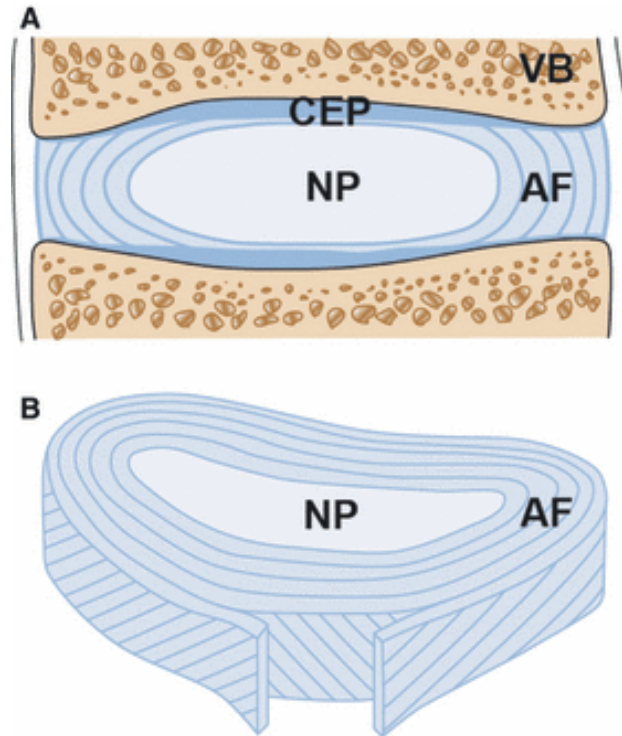
Figure 1. Schematic representation of an IVD. A. Sagittal section. The CEP and the vertebrae (VB) are visualized. B. The concentric lamellae that form the AF surround the NP region(Lotz, et al., 2002)
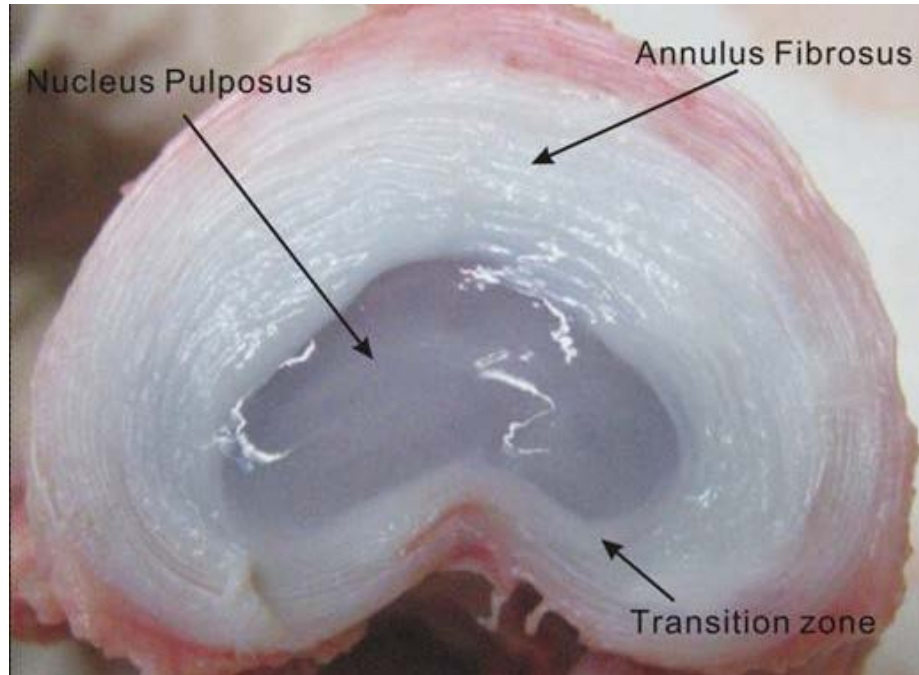
Figure 2. Transverse view of a porcine lumbar disc

The interplay of the two ECM main macromolecular components, the highly hydrated proteoglycan (PG) gel and the fibrillar collagen network, determine the disc's mechanical response. Collagen is responsible for the tensile strength of the disc, while PGs provide stiffness and resilience to compression through their interaction with water (Buckwalter, 1995). The PG comprises 50% of the dry weight in the nucleus and 20% in the annulus. Whereas, type I and II collagen fibrils make up 20% of the nucleus and 70% of the annulus dry weight (Buckwalter, 1995, Urban, 2003). Table 1 summarizes the proportions of water, collagen and PGs in the IVD.

Composition	IVD	AF	NP
Water (% wet wt.)	65-90%	60-70%	70-90%
Collagen (% dry wt.)	10-60%	50-70%	15-25%
Proteoglycans (% dry wt.)	15-65%	10-20%	~ 50%

Table 1. The composition of the human IVD. The IVD is comprised mostly of water with varying amounts of collagen and proteoglycan, depending on the location within the IVD. Fluid and PG concentrations are highest in the nucleus and lowest in the outer annulus

The concentration of collagens and PGs varies along different regions of the IVD. The outer annulus is composed mostly of type I collagen fibrils along with small amounts of type V collagen (80% and 3% respectively of the total collagen of the outer annulus) (Buckwalter, 1995). Furthermore, the concentration of type II collagen and PG increases towards the nucleus as the concentration of type I collagen falls. Type II collagen reaches 80% of the total collagen in the nucleus while small amounts of type XI collagen are found (3%) and type I collagen is absent in the nucleus. Type IX collagen is present in both, the nucleus and the annulus (less than 2%). In addition, type VI collagen is found in the annulus (10%) and the nucleus (above 15%) (Buckwalter, 1995). Among the diverse type of collagens present in the IVD, type I and II are the most abundant (80% of the total collagen) (Roughley, 1976). The increased hydroxylation of proline and lysine and the great occurrence of galactosyl-glucose substitution of the hydroxylysine are suggested to limit the lateral growth of collagen fibrils and to provide resilience to digestion by collagenases (Roughley, 1976). Table 2 shows the distribution of collagen types in the IVD.

Collagen type		Distribution	Percent of total collagen
Fibrillar	Type I	Outer and inner annulus, transition zone	Decreases from 80% to 0, from outer annulus to nucleus
	Type II	Nucleus, transition zone, inner annulus	Increases from 0 to 80% from outer annulus to nucleus
	Type III	Possible traces throughout disc	
	Type V	Outer and inner annulus	About 3%
	Type XI	Nucleus pulposus	About 3%
Short helix	Type VI	Annulus, nucleus	About 10%, more than 15% respectively%
	Type IX	Throughout disc	2% or less

Table 2. Distribution of collagen types in the IVD (Buckwalter, 1995)

Proteoglycans are present in the NP and the AF regions of IVDs and consist of a protein backbone to which glycosaminoglycans chains are covalently attached (Lundon and Bolton, 2001) (Figure 3). Aggrecan, versican, decorin, biblycan, fibromodulin, lumican and perlecan are some of the proteoglycans found in the IVD (Urban, 2003). Aggrecan is the most abundant PG on a weigh basis and it is present in the nucleus and the annulus. Table 3 shows the distribution of collagens and PG types in the IVD. Chondroitin sulfate and keratin sulfate are glycosaminoglycans chains attached to the core protein of aggrecan. At birth, chondroitin sulfate is the predominant chain bound to aggrecan, whereas the amount of keratin sulfate chains increases as the disc reaches maturity. Normal age related changes may be involved in the decrease in the length of chondroitin sulfate chains and the increase in the length of keratin sulfate chains. It is suggested that the growing of the disc and loss of vascularity are responsible for the decrease in oxygen supply that accompanies the variation in chain lengths (Roughley, 1976). Glucoronic acid, needed for the synthesis of chondroitin sulfate, is produced by oxidation, while keratin sulfate relies on galactose to be synthesized. The reciprocal variation of chain lengths is an attempt to preserve the high charge density in the aggrecan, which is needed for the osmotic properties of the disc (Roughley, 1976).
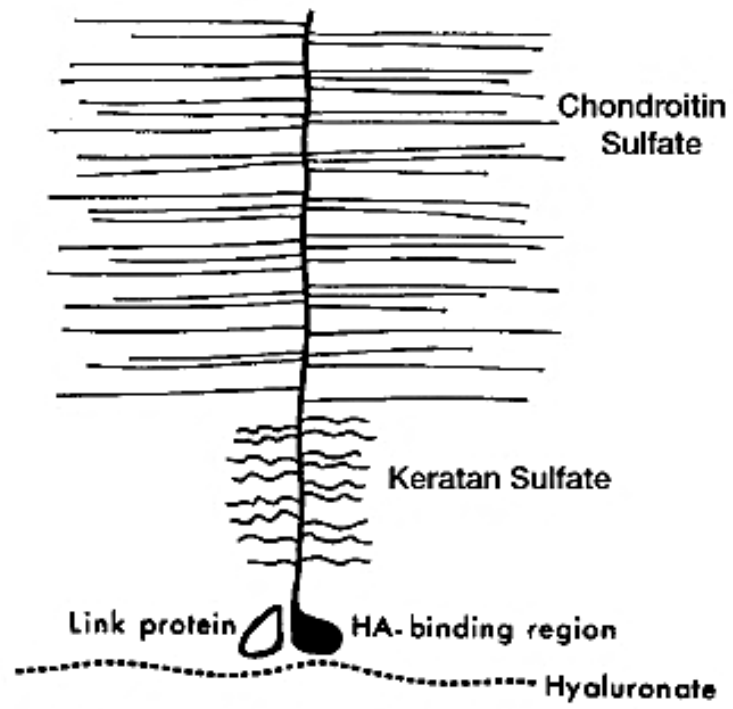
Figure 3. A proteoglycan unit (Guiot and Fessler, 2000)

Collagens		Proteoglycans	
Fibrillar	Type I	Aggregating	Aggrecan
	Type II		Versican
	Type III		Hyaluronan
	Type V		Link protein
	Type XI	Fibril-associated	Decorin
Fibril-associated	Type IX		Biblycan
	Type XII		Fibromodulin
	Type XIV		Lumican
Pericellular	Type VI	Pericellular	Perlecan
	Type X		

Table 3. Collagens and Proteoglycans in the disc extracellular matrix (Roughley, 1976)

2.1 CELLS FROM DIFFERENT REGIONS OF THE IVD

In the embryo, the NP is derived from the central notochord, while the mesenchyme forms the surrounding AF. The structure and composition of these two regions of the IVD are clearly seen in the embryonic disc (Roughley, 1976). The notochordal cells disappear by early adult life in humans but remains in some other species. In the postnatal NP, notochordal cells are spherical and large (30-40 µm in diameter) and have intracellular vacuoles that occupy about 25% of the cell area (Pattappa, et al., 2012). Extensive research on phenotype expression, surface markers and genome-wide microarray studies suggests that the mature NP cells are derived from notochordal cells (Fujita, et al., 2005, Power, et al., 2011). Cytokeratin -8, -18, -19 and brachyury or T-Box gene have been reported to be expressed by notochordal and mature NP cells. Further studies estate that brachyury expression is essential for notochordal cells differentiation (Takahashi, et al., 1999). In addition, it has been found that the signaling pathway through the expression of Sonic Hedgehog genes by notochordal cells is responsible for forming the NP (Choi, et al., 2008). Additionally, the use of fluorescence-activated cell sorting, combined with auto-fluorescence and size analysis, has revealed that notochordal-like cells express protein integrin subunits that modulate interactions with collagens and laminin but lack gene expression for specific small proteoglycans compared to NP cells (Chen, et al., 2006). In contrast to notochordal cells, NP cells are smaller (10 µm in diameter), round and without intracellular vacuoles (Pattappa, et al., 2012). They are found in the mature disc at a density of 4×10^6 cells/cm^3 (Roughley, 1976). NP specific markers have been identified with the aid of microarray studies on the transcriptome of the NP cells. Further research have

reported the expression of chondrocyte markers like SOX-9 and type II collagen in NP cells from healthy and degenerative discs (Sive, et al., 2002). Fluorescence-activated cell sorting showed that cells from the immature NP were larger and with higher fluorescence than those in the AF region (Chen, et al., 2006). Compared to NP cells, AF cells are thin, elongated and resemble fibroblasts. In the mature AF, the cell density is about 9×10^6 cells/cm^3, with a higher density in the outer layers than in the inner annulus (Hastreiter, et al., 2001). AF phenotypic markers have not been clearly identified since variability of gene expression is found in different cell types and species (Sakai, et al., 1976). However, further studies addressing this issue have showed tenomodulin as a potential AF marker in bovine and human species with increased gene expression in degenerated AF cells (Minogue, et al., 2010). Figure 4 shows the morphology differences between NP and AF cells seeded in agarose gel constructs.
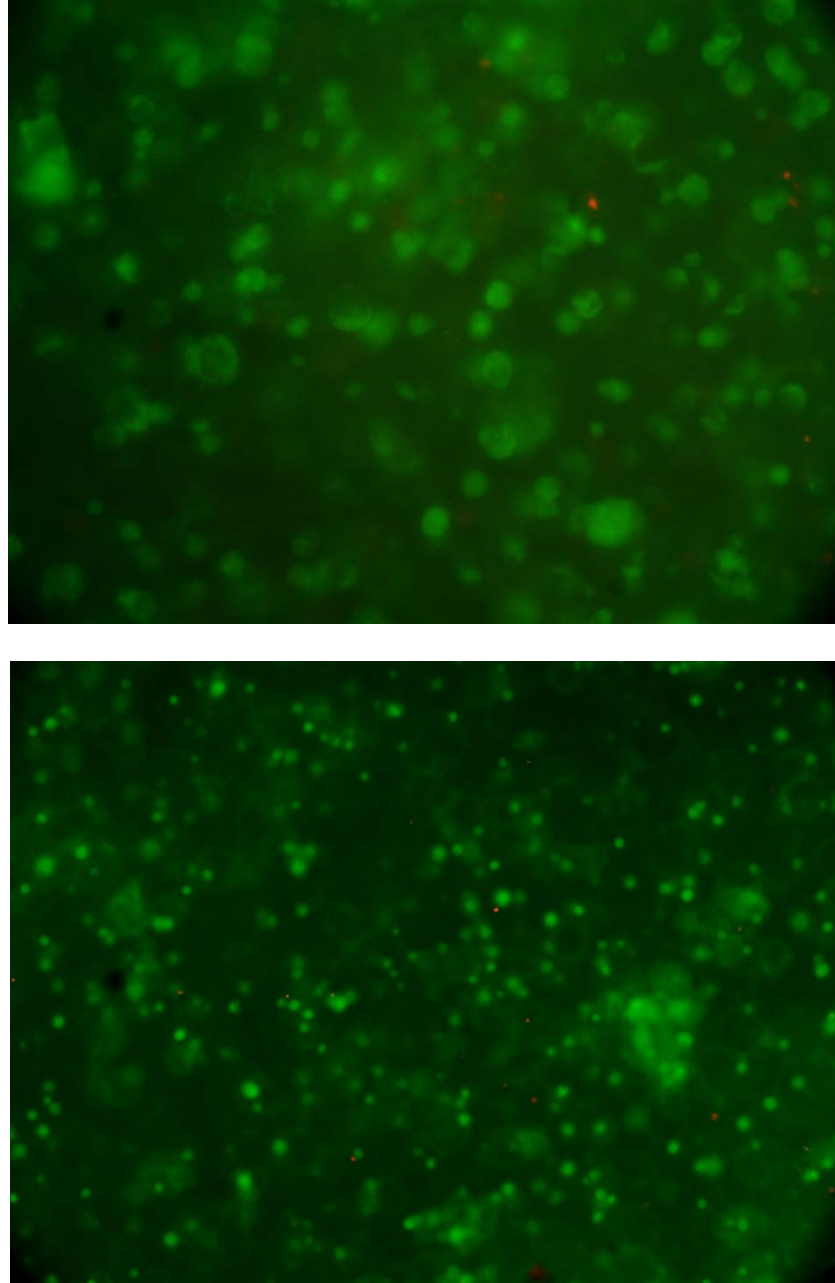
Figure 4. Cell viability staining of NP cells (top) and AF cells (bottom). Live cells are stained in green, dead cells are stained in red. Magnification: 20X for both pictures

2.2 IVD DEGENERATION

It is well- established that modifications in the IVD composition due to pathological or aging changes result in the decline of the biochemical and biomechanical properties of the IVD (Inoue and Espinoza Orias, 2011, Roughley, 1976). Remodeling, breakdown, and rearrangement of the ECM are normal processes of healthy discs environment that in some cases can lead to IVD degeneration (Maclean, et al., 2004). The reduction of cell number and loss of key matrix molecules such as proteoglycans and collagens is markedly accelerated with age and disc deterioration (Preradovic, et al., 2005, Walker and Anderson, 2004). It has been reported that cells from the NP and AF region respond differently to tissue degeneration (Cs-Szabo, et al., 1976). Cells from the NP show decreased mRNA expression and declined protein content of proteoglycans, whereas cells from the AF show upregulation of biosynthetic processes at an early stage of degeneration but a continuous decline at advance stages (Cs-Szabo, et al., 1976). Therefore, it is suggested that the structural and compositional differences of NP and AF make the latter less vulnerable to degeneration than the nucleus region.

Normal aging changes in the human IVD structure are depicted in Figure 5. The IVDs from the fetal, the juvenile, and the adult individuals are bordered laterally by the ligaments of the spine and axially by the cartilaginous endplates of the vertebrae. The fetus presents wide vascular endplates that cover the whole IVD. The NP is jelly-like and rich in PGs and notochordal cells, and the lamellar AF is vascular. In the juvenile, the endplates have reduced their thickness and are less vascular. The AF is poorly vascularized and the NP has more collagen content. The notochordal cells decline in number and are replaced by

mature NP cells. In the adult, the narrow endplates do not cover the entire nucleus of the IVD and may be calcified. Moreover, the NP becomes less distinct from the inner annulus and the notochordal cells disappear (Roughley, 1976).
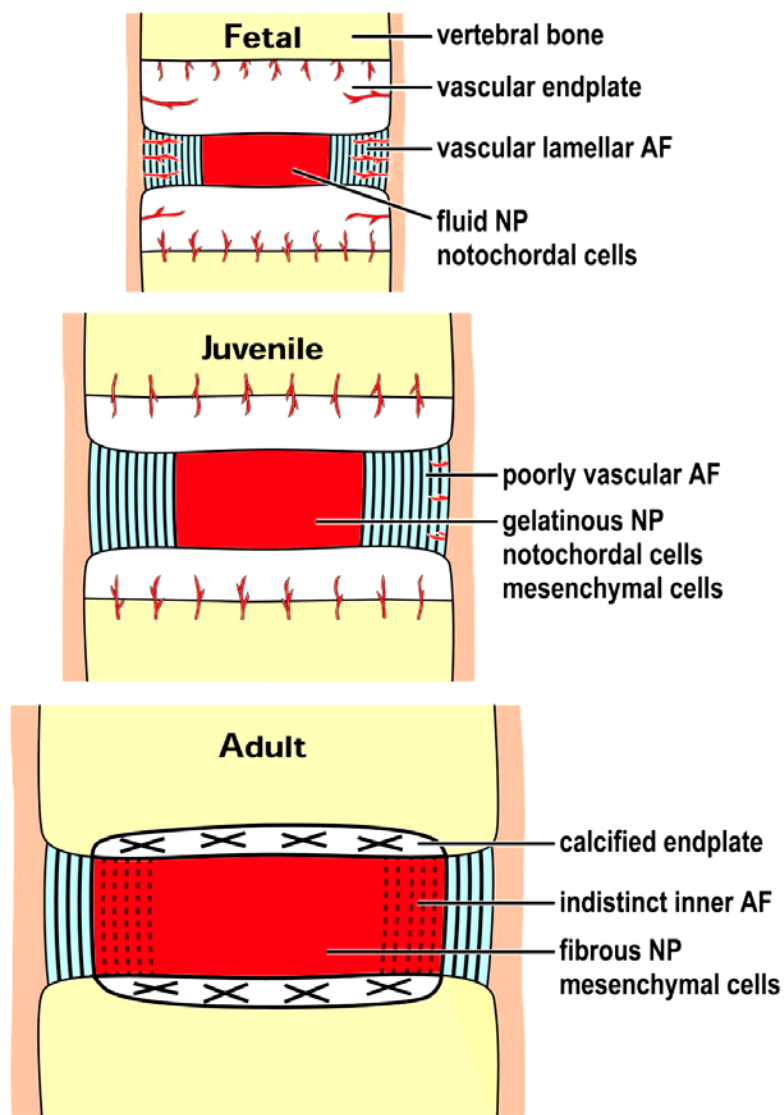
Figure 5. Variation in human intervertebral disc structure with age (Roughley, 1976)

The composition of the human NP also changes with age. Figure 6 depicts the changes in collagen fibrils and aggrecan-derived PGs in the NP region of the fetus, the young juvenile, the adolescent/young adult and the mature adult/degenerated IVD. The aggrecan in the fetus is rich in chondroitin sulfate and presents little collagen content. In the young juvenile, the aggrecan has chondroitin sulfate and keratin sulfate chains attached to its protein core and the collagen content is higher. Whereas in the young adult NP, the aggrecan and link protein have experienced proteolytic damage and the chondroitin sulfate chains of aggrecan are smaller while its keratin sulfate chains are bigger. In addition, the collagen content reaches its maximum value. In the mature or degenerated NP, the aggrecan, link protein and collagen fibrils show enhanced proteolytic damage. Also, the levels of no aggregated aggrecan and hyaluronan are increased (Roughley, 1976).

The synthetic capabilities of IVD cells may be impaired by notochordal cell depletion, aging of cells and decline in cell density. At the same time, structural changes such as vascular depletion, endplate calcification and growing of the disc contribute to decreased nutrient supply and accumulation of degradation products. While all the aforementioned changes can be attributed to normal processes in the IVD, adverse compressive and shear loading can also alter cell metabolism and thus, ECM integrity. Defective matrix structures as a consequence of genetic alterations can also lead to IVDs more susceptible to disc degeneration (Roughley, 1976). Figure 7 is a comparison of young and healthy and severely degenerated discs.
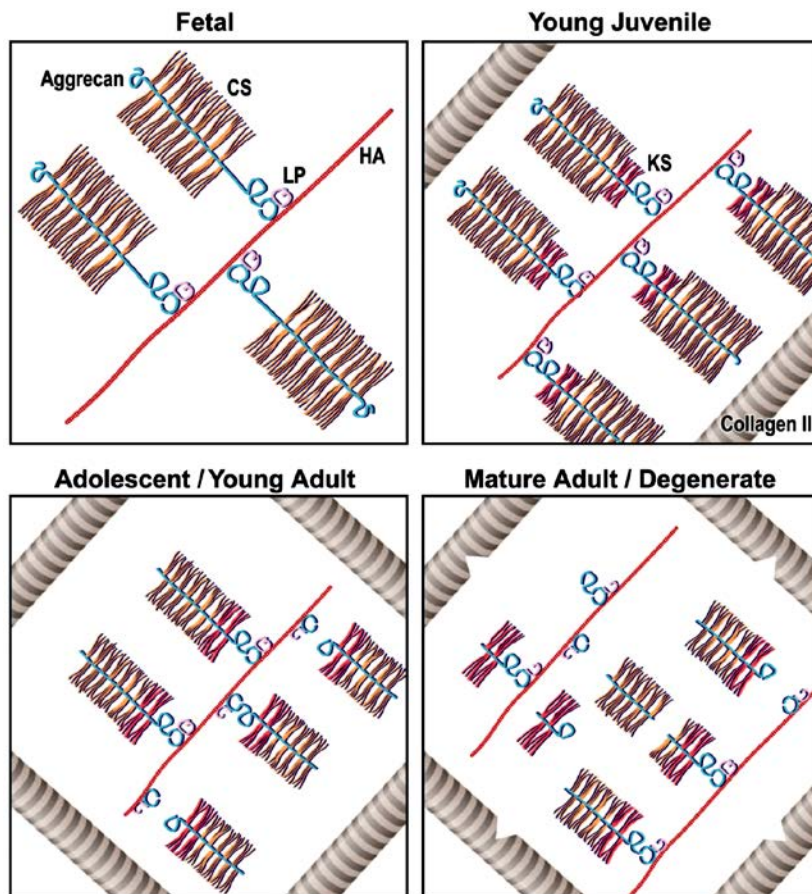
Figure 6. Collagen fibrils and aggrecan-derived proteoglycans in the NP of fetal, young juvenile, adolescent and mature adult IVDs (Roughley, 1976)
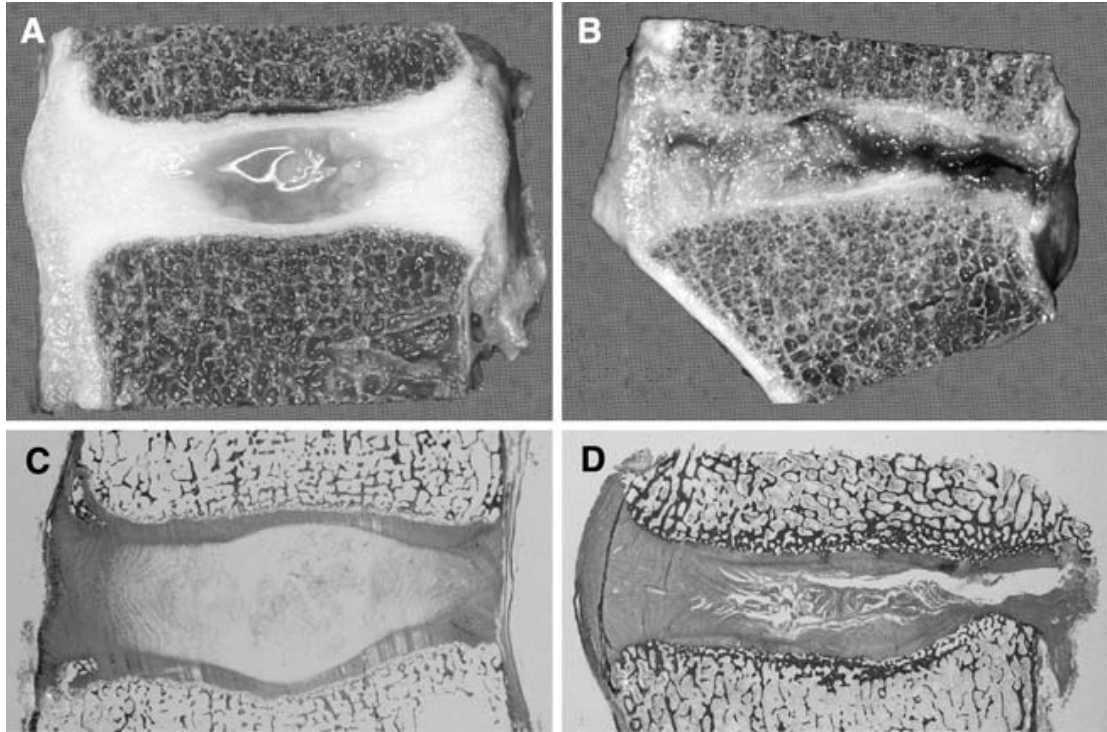
Figure 7. Comparison of young and healthy (A and C) and severely degenerated (B and D) discs at the macroscopical (A and B) and the histological (C and D) levels (Paesold, et al., 2007)

2.3 ADENOSINE

The sources of adenosine include its release as a result of cell damage or through transporters in order to maintain equilibrium or the hydrolysis of extracellular adenine nucleotides (e.g. extracellular ATP) by the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family (Robson, et al., 2006, Zimmermann, 2000), the ecto-nucleotide pyrophosphatase/ phosphodiesterase (E-NPP) family (Goding, et al., 2003, Stefan, et al., 2006), ecto-5'-nucleotidase/CD 73 (Colgan, et al., 2006, Hunsucker, et al., 2005, Zimmermann, 1992), and alkaline phosphatases (AP) (Millán). Adenosine has shown to increase the ratio of oxygen supply to demand, to protect against ischemic damage, to trigger anti-inflammatory responses and to promote angiogenesis (Jacobson and Gao, 2006). In addition, high adenosine concentrations have been found in ischemic or inflamed tissues (Cronstein, et al., 1993, Matherne, et al., 1990).

With adenosine receptors expressed in most cells and organs, overwhelming evidence propose to target adenosine receptors to treat diverse conditions such as cerebral and cardiac ischemic diseases, sleep disorders, immune and inflammatory disorders and cancer (Jacobson and Gao, 2006). When exposed to extracellular ATP, ATP receptors will be activated, but as the ATP is progressively metabolized to adenosine, adenosine receptors will subsequently be activated too (Dubyak and el-Moatassim, 1993). Adenosine receptors, also known as P1 receptors, are part of the G protein coupled receptors family and are classified into four subtypes with unique pharmacological characteristics. The A1 and A3 receptors are $G_{i/o}$ types that have 49% of sequence similarity in humans and decrease intracellular cAMP levels (Smyth and Stagg, 2010). The A_{2A} and A_{2B} are G_s-coupled that

have 59% of sequence similarity in humans and increase the cAMP levels through the activation of adenylyl cyclase (Haskó and Cronstein, 2004).

2.4 SIGNIFICANCE OF THIS STUDY

Disc degeneration is associated with low back pain which afflicts millions of people in the US. The IVD, along with the facets and ligaments, contributes to the transmission of loads and the functional movement of the spine. The mechanical properties of the IVD rely on the composition and organization of its ECM. Improper biosynthesis, breakdown and accumulation of ECM constituents due to aging or/and degeneration can be factors involved in the quality and integrity of the ECM. Recent studies have demonstrated that mechanical loading promotes ATP production and release from IVD cells (Czamanski, et al., 2011, Fernando, et al., 2011) and ECM biosynthesis in chondrocytes (Chowdhury and Knight, 2006). Since ATP is a powerful signaling molecule which can regulate a variety of biological processes, the mechanosensitive ATP release found in a previous study may be involved in the process of mechanotransduction of IVD cells. Furthermore, high accumulation of extracellular ATP found in the NP region (Wang, et al., 2013) suggests that ATP is key in the maintenance of a healthy ECM structure in the IVD. Hence, by stimulating the underlying mechanotransduction purinergic pathway using ATP, it may be possible to obtain the same cellular response without externally applied forces, which could overcome the problem of mechanically stimulating tissues with irregular geometry. It was also demonstrated that mechanical loading decreases extracellular ATP content in the NP (Wang, et al., 2013), which suggests that compressive loading promotes hydrolysis of ATP. Not only ATP but also adenosine, a byproduct of ATP hydrolysis, has proven to be

involved in signaling roles in a variety of tissues (Dubyak and el-Moatassim, 1993). However, no studies have been conducted to investigate the effects that stimulation of the purinergic pathway elicit on the ECM synthesis of IVD cells. Therefore, the objective of this study was to investigate the effects of direct stimulation of the purinergic pathway by extracellular ATP and adenosine on the modulation of the ECM synthesis in IVD cells. The results of this study will provide a better understanding of the ECM synthesis process in the IVD towards the designing of new treatment strategies to address disc degeneration.

CHAPTER 3: MEASUREMENT OF ATP-INDUCED MEMBRANE POTENTIAL CHANGES IN IVD CELLS

3.1 INTRODUCTORY REMARKS

In addition to its role as an intracellular energy source, ATP is an extracellular signaling molecule actively involved in the regulation of short-term and long-term processes through the modification of proliferation, differentiation, and apoptosis of cells via purinergic pathways (Burnstock, 1997). The receptors involved in the transduction of purinergic signals are distributed in a wide variety of tissues throughout the body and therefore, biological responses to extracellular ATP have been reported in tissues as diverse as skin, skeletal muscle, bone and the immune system (Burnstock and Knight, 2004). In previous studies of cells and tissues that exhibited a biological response to extracellular ATP, signaling cascades that affected the cell membrane potential and excitability were activated by extracellular ATP (Dubyak and el-Moatassim, 1993).

The IVD cells maintain the proper homeostatic balance of biosynthesis, breakdown, and accumulation of ECM molecules (Ohshima, et al., 1995). These cellular processes determine the quality and integrity of the ECM and thus, the mechanical response of the IVD (Buschmann, et al., 1995). Recent studies have demonstrated that compressive loading affects ATP production and release by IVD cells in the 3-dimensional agarose gel model (Czamanski, et al., 2011, Fernando, et al., 2011) and in-situ energy metabolism in the IVD (Wang, et al., 2013).

A high accumulated level of extracellular ATP (~165 µM) compared to physiological concentrations (1-50 nM)(Trabanelli, et al., 2012) has been found in the NP of the IVD (Wang, et al., 2013). However, whether extracellular ATP can induce a signaling response in IVD cells has not been investigated.

Membrane potentials are the difference in electric potential across the bilayer membrane of a cell (Sundelacruz, et al., 2009). The combined actions of ion channels and transporters establish membrane potentials by regulating intracellular and extracellular ionic concentrations(Sundelacruz, et al., 2009, Yang and Brackenbury, 2013). Increasing evidence suggests that membrane potentials are not only involved in the cellular processes of proliferation, migration and differentiation (Binggeli and Weinstein, 1986, Blackiston, et al., 2009, Sundelacruz, et al., 2013), but are also related to complex processes such as control of wound healing (Nuccitelli, 2003a, Nuccitelli, 2003b), regeneration (Levin, 2007, Levin, 2009), left-right patterning (Levin, et al., 2002) and development (Nuccitelli, 2003a). Thus, it is suggested that membrane potentials are a fundamental control mechanism of cell functions (Sundelacruz, et al., 2009). While patch clamp techniques are successfully and widely used to control and measure membrane potentials, system set-up is laborious and requires extensive expertise (Sundelacruz, et al., 2009). Another approach to monitor membrane potentials is the use of potential sensitive dyes, which provide ease of use and a noninvasive set up. The aminonaphthylethenylpyridinium (ANEP) group of dyes offers rapid shifts in their spectra in response to changes in the surrounding electric field and modify their electronic structure, thus allowing fast optical responses (in the millisecond-range) to membrane potential changes (Invitrogen, 2006). Among the ANEP

dyes, di-8-ANEPPS is more appropriate for long term experiments because the dye is maintained in the outer leaflet of the plasma membrane due to its lipophilic property (Biotium). Upon binding to the cell membrane, di-8- ANEPPS becomes strongly fluorescent. A membrane potential change is followed by a redistribution of the intramolecular charge. Consequently, the spectral profile and fluorescence intensity of di-8- ANEPPS change, and the variations of fluorescence intensity are proportional to variations in membrane potential (Pucihar, et al., 2009). Di-8-ANEPPS has been used to measure membrane potentials induced by electrical stimulation (Pucihar, et al., 2009). It has also been used to complement electrophysiological techniques(DiFranco, et al., 2005, Zhang, et al., 1998). However, to our knowledge, noninvasive measurements of transient membrane potential changes induced by exogenous ATP using di-8-ANEPPS have not been reported.

3.2 MATERIALS AND METHODS

Sample preparation

IVD tissues from the NP and the AF regions were harvested from the spine of mature pigs (~ 250 lbs.) within 2 hours of sacrifice (Cabrera Farms, Hialeah, FL). The IVD tissues were enzymatically digested in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corp., Carlsbad, CA) containing 1 mg/mL collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) and 0.6 mg/mL protease (Sigma Aldrich, St. Louis, MO) for about 24 hours at 37 °C, 5% CO_2. AF tissue required to be broken down mechanically using a syringe to facilitate tissue digestion while in its respective digestion solution. The mixtures containing IVD cells were filtered using a 70μm strainer (BD Biosciences, San Jose, CA)

to remove undigested tissue. The cells were re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp.) and 1% antibiotic-antimycotic and were mixed at a 1:1 ratio with 4% agarose gel. Cell-agarose constructs were made by casting 100μL of the cell-agarose mixture in a custom-made mold to obtain a construct of 1×10^6 cells in 2% agarose (Figure 8). The constructs were incubated overnight at 37°C, 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. Additionally, some NP and AF cells were expanded in monolayer culture. The cells of primary culture were plated on cover slides. The samples, either cells seeded in agarose or cells plated on cover slides, were placed in a RC-21BR large volume closed bath imaging chamber (Warner Instruments, Hamden, CT) (Figure 9). The samples were washed with phosphate buffered saline (PBS; Lonza, Walkersville, MD) for 5 minutes at 0.3 mL/min using a syringe pump (Harvard Apparatus, Holliston, MA) and then were incubated for 10 minutes (cells on cover slides) or 15 minutes (cells seeded in agarose) at 4°C in a staining solution, which is PBS containing 30 μM of di-8-ANEPPS and 0.05% of Pluronic F-127 (both Biotium, Hayward, CA) (Fromherz, et al., 2008). Uneven membrane staining and progressive dye internalization prevent di-8- ANEPPS from being used for absolute measurements such as resting membrane potential unless measured in conjunction with microelectrodes (Beach, et al., 1996, Knisley, et al., 2000, Pucihar, et al., 2009). In this study, uneven membrane staining was improved by using Pluronic F-127 (0.05%) to aid solubilization of the dye and dye internalization was inhibited by incubation of the samples at 4°C (Pucihar, et al., 2009). The staining solution was gently washed away with PBS for 5 minutes at 0.3 mL/min; a single cell was selected and observed using a fluorescence

microscope (Olympus IX70, Melville, NY) equipped with a 20X objective (LCPlan FL, 0.60 Ph2, Olympus), an advanced fluorescence illuminator (Lumen 200, Prior Scientific Inc., Rockland, MA), a filter for di-8-ANEPPS (QD625-A-OMF, Semrock, Rochester, NY (excitation: 435 nm, emission: 625 nm)) and a digital CCD camera (Retiga 2000R, Q-Imaging, Canada) (Figure 10). In the 3-dimensional culture, a cell located on the periphery of the gel was selected in order to prevent any time delay of the response due to ATP diffusion in agarose gel.

Figure 8. 2% agarose disc (8 mm in diameter and 2mm in thickness)

Figure 9. RC-21BR large volume closed bath imaging chamber (Warner Instruments, Hamden, CT) with syringe used for PBS infusion attached
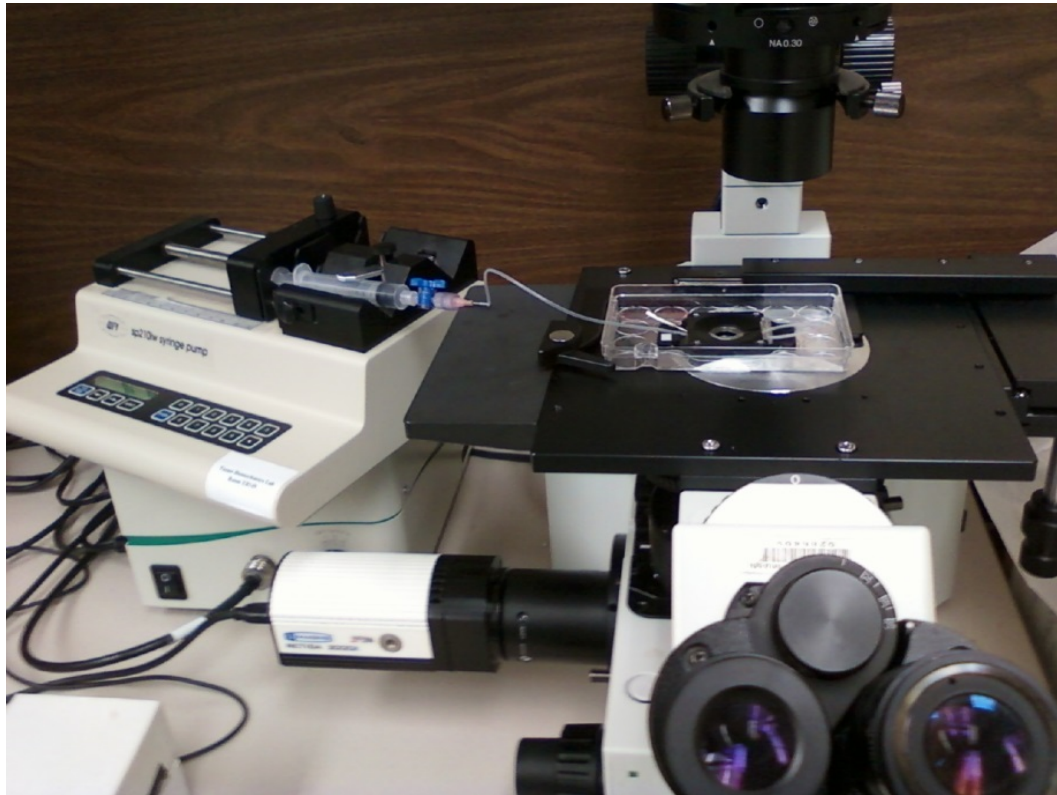
Figure 10. ATP injection setting. The pump infuses ATP to the bath imaging chamber (1.6 mL/min for 10 seconds) and the cell response is recorded in a movie structure for further analysis

ATP injection

ATP at either 100, 200, 300 or 500 µM concentration was injected into the chamber at 1.6 mL/min for 10 seconds. The time and rate of injection were selected to ensure that the contents of the chamber (260 µl volume) were substituted by the injected ATP. A sequence of fluorescence images was recorded starting at 10 seconds before ATP injection and continued during and after the injection for a total of 50 seconds. The sequence of fluorescence images was acquired in a movie structure at a rate of over 120 frames per second using QCapture Pro v6 (Media Cybernetics Inc. and Q-Imaging Inc., Canada) for further image processing.

Data processing

The cell membrane was labeled by a program implemented in MATLAB v7.1 (The MathWorks Inc., Natick, MA). Briefly, noise was reduced using blind deconvolution on every image in the recording sequence (Pucihar, et al., 2009). Then, the first image in the sequence was used as a reference to obtain an intensity threshold level used to accentuate the ring stain pattern defining the cell membrane. A binary image of the cell membrane was then created from the first image based on the defined intensity threshold level (Zhang, et al., 1998). Finally, all the images in the recording sequence were multiplied by the binary image in order to label the same region of interest on the cell membrane (Figure 11).
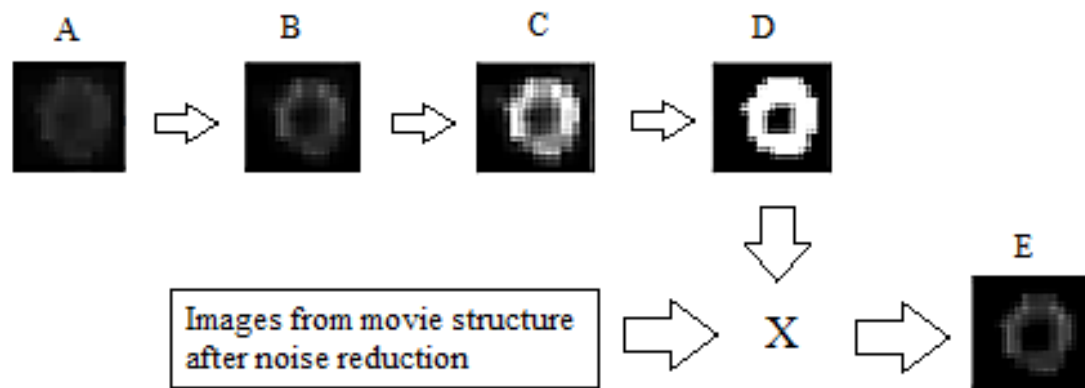
Figure 11. Image processing procedure. A. first image of the recording sequence. B. blind deconvolution. C. gray level adjustment. D. binary image creation. E. example of a resultant image

After image processing, the average intensity of the cell membrane was computed for images at all time-points and was then normalized by the average intensity of the cell membrane before ATP response. A normalized intensity levels vs. time curve, which exhibited the time decay of intensity due to photobleaching, was obtained (Figure 12A). An exponential model $I = ae^{bt} + ce^{dt}$, where I is the normalized intensity, t is the time, and a, b, c and d are the coefficients, was used to describe the time decay of fluorescence intensity (Figure 12B). The coefficients were determined by curve fitting the theoretical model with the experimental intensity curve (Figure 12B). Then, the experimental intensity curve was compensated using the theoretical model (Figure 12C). Finally, the compensated experimental intensity curve was filtered with a 4th order low pass Butterworth filter with a cutoff frequency of 15 Hz to yield a smooth curve (Figure 12D). The increase in membrane potential in response to ATP stimulation was expressed as a percentage compared to the normalized average intensity of the cell membrane before ATP response.
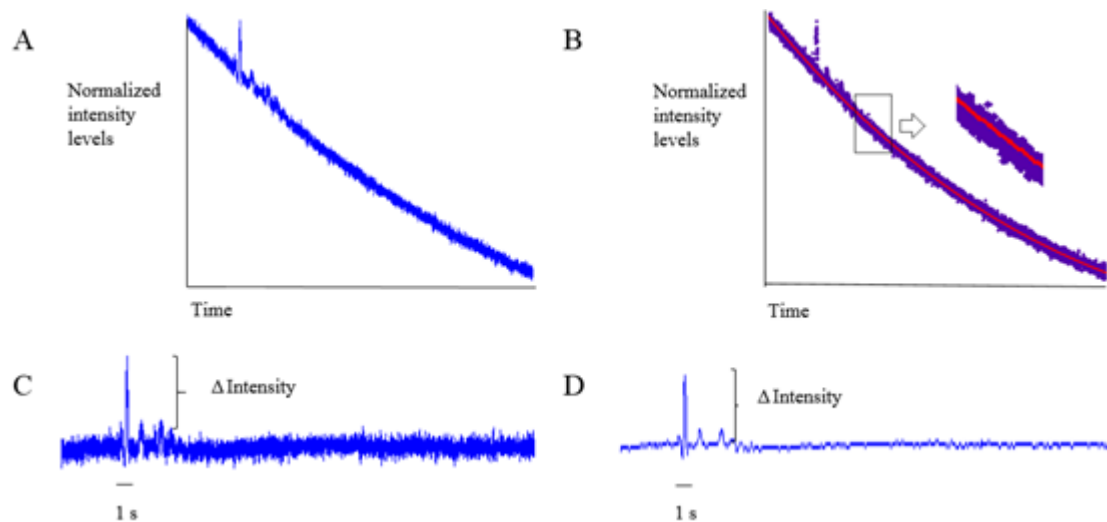
Figure 12. Compensation procedure for the time decay of fluorescence intensity due to photobleaching. A. a typical original experimental intensity curve showing the photobleaching effect. B. theoretical curve fitting (the red curve is an exponential model). C. the experimental intensity curve in (A) compensated by the exponential model obtained in (B). D. the compensated intensity curve after filtering

Gene expression of purinergic receptors

Total RNA from IVD cells in monolayer and 3-dimensional agarose gel cultures was extracted using the trizol (Tri-Reagent, Molecular Research Center, Cincinnati, OH) protocol. Total RNA was quantified using the Qubit RNA BR assay kit (Life Technologies, Carlsbad, CA) and reverse transcribed to cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA), according to the manufacturers' specifications. mRNA expression of $P2X_4$ purinergic receptor and 18S (endogenous control) was examined using SYBR Green Super Mix ROX (Quanta Biosciences, Gaithersburg, MD) and real-time RT-PCR (One step Plus, Applied Biosystems). The PCR products were electrophoresed on a 2% ethidium bromide-stained agarose gel in order to examine the expression of the purinergic receptor. The primer sequences were as follow: $P2X_4$ forward primer: CCGGGACGTGGACCACAACG; $P2X_4$ reverse primer: TGCGGCATGGGCTTCACTGG; 18S forward primer: CGGCTACCACATCCAAGGA; 18S reverse primer: AGCTGGAATTACCGCGGCT. The sizes of PCR products for $P2X_4$ and 18S were 245 and 188 bp, respectively.

Inhibition of P2 receptors

The involvement of the purinergic pathway in the cell membrane depolarization response was studied using pyridoxalphosphate-6-azophenyl-2', 4'-disulfonate (PPADS), a non-competitive inhibitor of P2 receptors, on IVD cells cultured on cover slides. Before an inhibitor treatment, the samples were washed with PBS for 5 minutes at 0.3 mL/min and then incubated at room temperature in PBS for an additional 5 minutes. Then, the samples

were incubated with PBS containing 3, 10 or 30 µM of PPADS at room temperature for 20 minutes. After the inhibitor treatment, 500 µM ATP was injected to the chamber at 1.6 mL/min for 10 seconds while fluorescence images were acquired for further processing.

Statistical analysis

Student's t-test analyses were performed using Excel data analysis tool (Microsoft, Seattle, WA) to compare the magnitude of the response between different concentrations of ATP under the same culture conditions for each cell type and to compare the magnitude of the response between NP and AF cells under the same culture conditions and concentrations of ATP. Additionally, a way ANOVA followed by a post hoc Student Newman Keuls test (SPSS Statistics 20, Chicago, IL) was performed to compare differences in the response of IVD cells to 3, 10 or 30 µM of PPADS. Differences in all cases were considered significant at $p < 0.05$.

3.3 RESULTS

AF cells

The membrane potential change response of AF cells in monolayer culture was a single spike (Figure 13A). The change in fluorescence intensity was 4.87% ± 0.83% for 200 µM ATP and 8.52% ± 1.93% for 500 µM ATP. In 3-dimensional agarose gel culture, the membrane potential change response was a wide upward deflection (Figure 13C) resulting in a change in fluorescence intensity of 3.7% ± 0.73% for 200 µM ATP and 7.98% ± 1.53% for 500 µM ATP (mean ± SEM; n = 5 for each measurement). The fluorescence intensity induced by 500 µM ATP was significantly higher than the 200 µM ATP in the 3-

dimensional culture (Figure 14A, p = 0.036). In both culture environments, no responses were observed from the 100 µM ATP injection. No responses were observed by injection of PBS only.
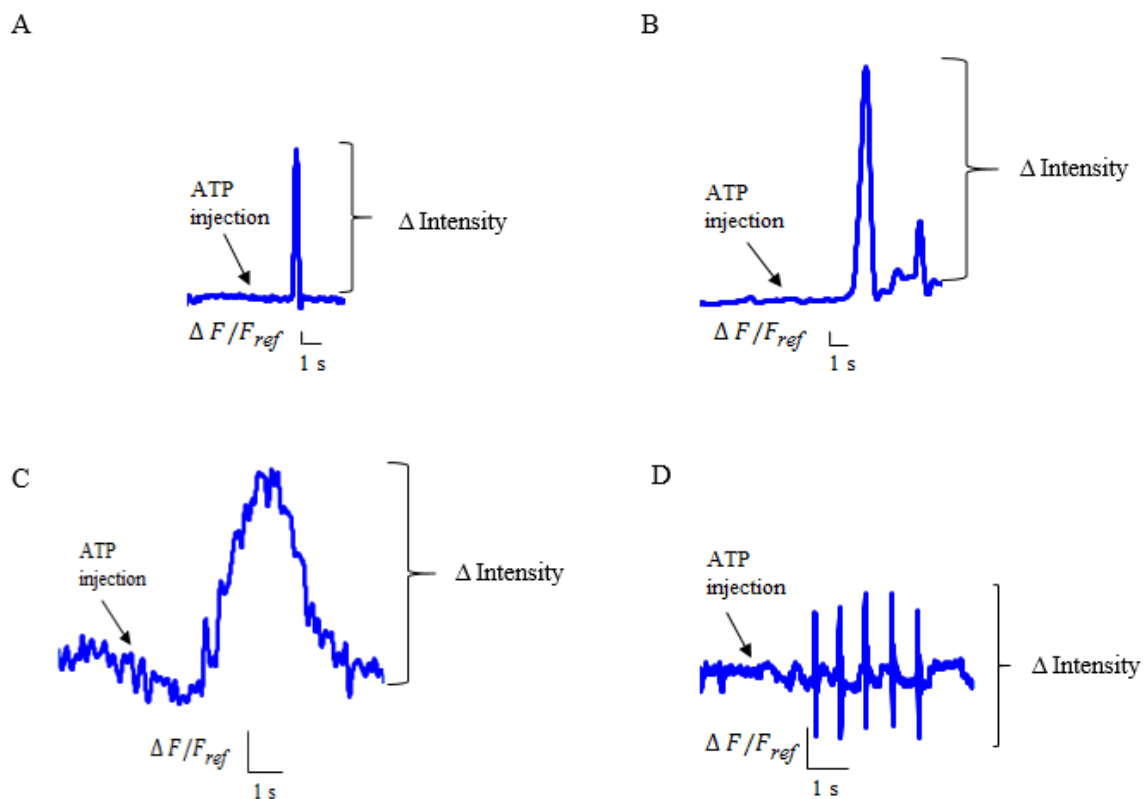
Figure 13. Typical responses in monolayer and 3-dimensional agarose gel cultures. A. fluorescence intensity change of the cell membrane in AF cells in monolayer culture induced by 500 μM ATP. B. fluorescence intensity change of the cell membrane in NP cells in monolayer culture induced by 500 μM ATP. C. fluorescence intensity change of the cell membrane in AF cells in 3-dimensional agarose gel culture induced by 500 μM ATP. D. fluorescence intensity change of the cell membrane in NP cells in 3-dimensional agarose gel culture induced by 500 μM ATP. $\Delta F/F_{ref}$ is the normalized change in fluorescence intensity. ΔF: Fluorescence variation, F_{ref}: Fluorescence of the first image
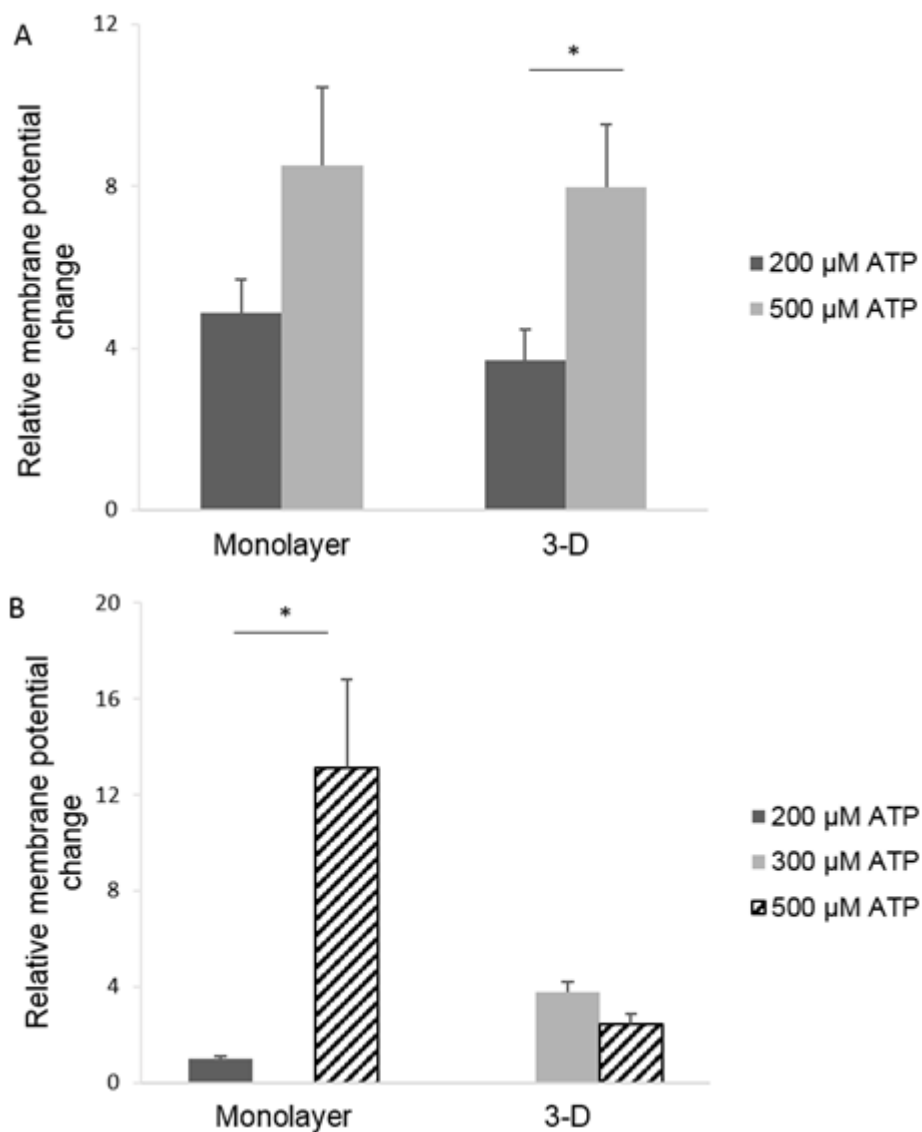
Figure 14. Comparison of the relative membrane potential change of IVD cells in monolayer and 3-dimensional cultures (n = 5 for each measurement). A. AF cells (Monolayer: p = 0.12, 3-D: p = 0.036). B. NP cells (Monolayer: p = 0.031, 3-D: p = 0.08)

NP cells

The membrane potential change response of NP cells in monolayer culture was a main spike followed by attenuated spikes (Figure 13B). The change in fluorescence intensity was 1% ± 0.08% for 200 µM ATP and 13.1% ± 3.74% for 500 µM ATP. The fluorescence intensity induced by 500 µM ATP was significantly higher than 200 µM ATP (Figure 14B, p = 0.031). No response was observed for the injection of 100 µM ATP. In the 3-dimensional culture, NP cells responded with several interspersed spikes (Figure 13D). The fluorescence intensity was altered by only the injection of 300 µM or 500 µM ATP, with the changes being 3.75% ± 0.46% for 300 µM ATP and 2.45% ± 0.42% for 500 µM ATP (mean ± SEM; n = 5 for each measurement). No responses were observed by injection of PBS only.

Comparison of monolayer and 3-dimensional agarose gel cultures

The fluorescence intensity induced by 200 µM ATP was significantly higher in AF cells compared to NP cells in monolayer culture (Figure 15A, p = 0.01). In 3-dimensional agarose gel culture, the fluorescence intensity induced by 500 µM ATP was significantly higher in AF cells compared to NP cells (Figure 15B, p = 0.017). It was also observed that ATP-induced responses had a longer duration in agarose culture than in monolayer culture. In addition, in both cell culture environments, ATP elicited a transient depolarization in which the membrane potential returned to its previous state while ATP was still present.
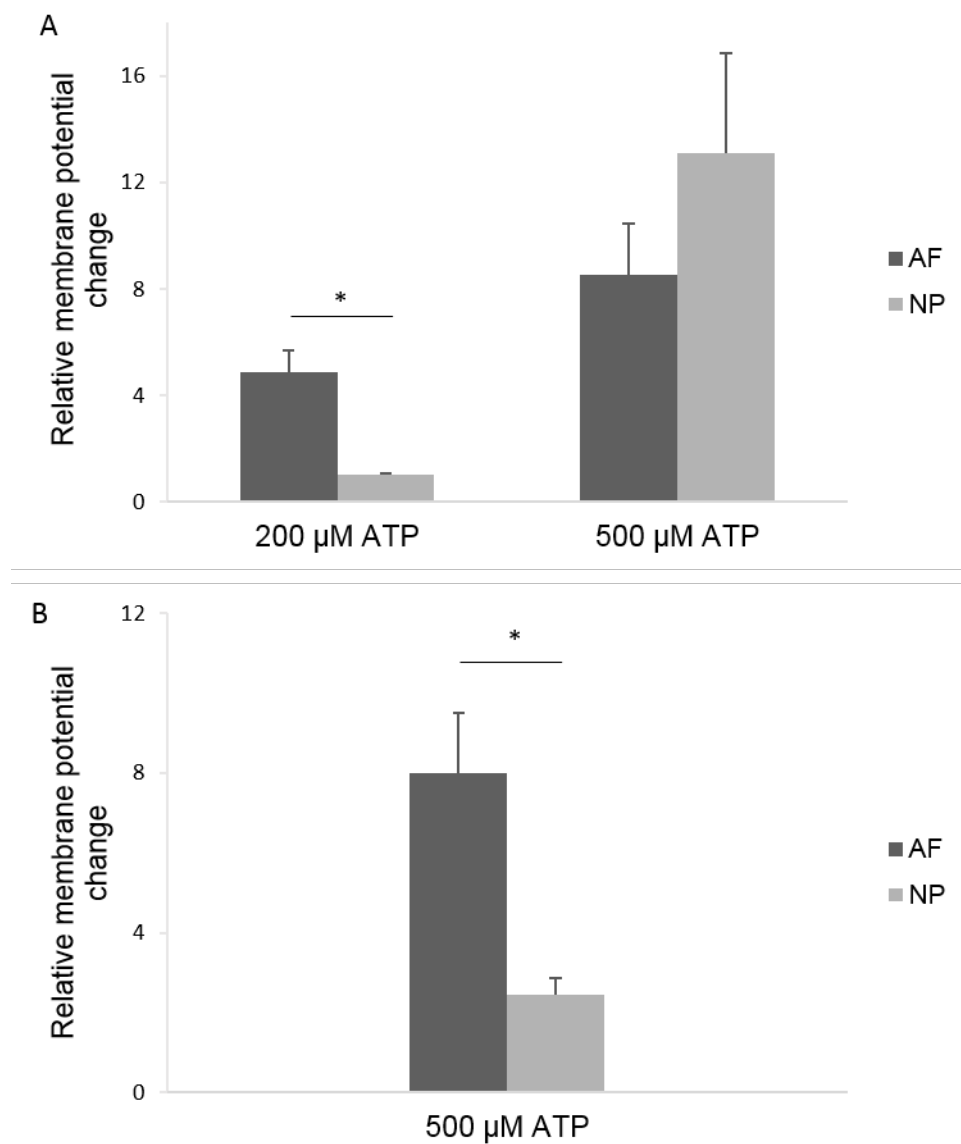
Figure 15. Comparison of the relative membrane potential change of IVD cells under different ATP concentrations (n = 5 for each measurement). A. Monolayer culture (200 µM: p = 0.01, 500 µM: p = 0.31). B. 3-dimensional agarose gel culture (500 µM: p = 0.017)

Gene expression of purinergic receptors

The gene of the P2X$_4$ purinergic receptor was expressed by AF and NP cells in monolayer and 3-dimensional agarose gel cultures (Figure 16).

Monolayer AF   3-dimensional AF

P2X$_4$

18S

Monolayer NP   3-dimensional NP
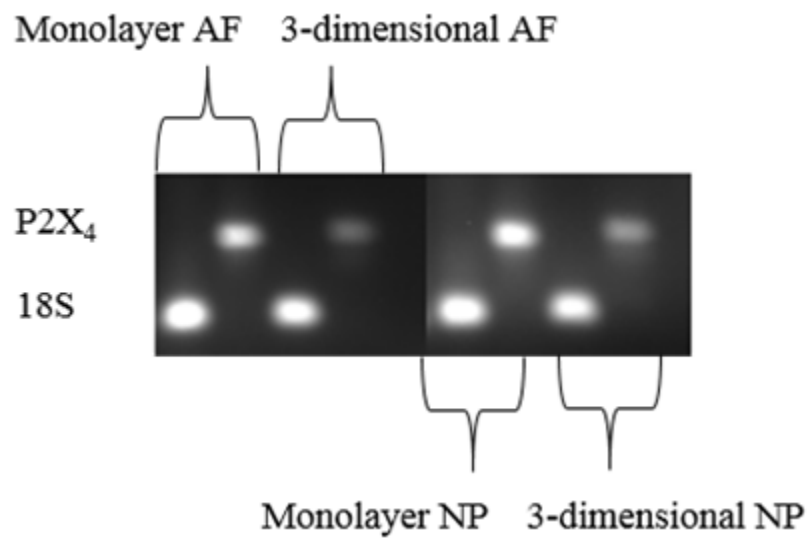
Figure 16. Gene expression of purinergic receptor P2X4 in AF and NP cells cultured in monolayer and 3-dimensional agarose gel. 18S is the endogenous control

Inhibition of P2 receptors

PPADS was used in the ATP injection experiments to examine whether the purinergic pathway has a role in the membrane potential change of IVD cells. No significant difference in the response of IVD cells treated with PPADS at 3, 10 or 30 μM of PPADS was found (AF: p = 0.569; NP: p = 0.454; n = 3 for each concentration of PPADS). Furthermore, a significant difference in response to ATP stimulation between IVD cells with and without PPADS was found, confirming that PPADS abolished the ATP-induced membrane potential response of IVD cells (AF: p = 0.013; NP: p = 0.032).

3.4 DISCUSSION

This study established a noninvasive system to measure ATP-induced membrane potential changes using the potentiometric dye di-8-ANEPPS and digital imaging processing techniques. Using this system, ATP-induced transient membrane potential changes of IVD cells were detected in both monolayer and 3-dimensional cultures. To our knowledge, this is the first study to show transient membrane potential changes that exogenous ATP triggers in IVD cells using a potentiometric dye.

Unlike electrodes, potentiometric dyes are easy to use and are free from electrical stimulus artifacts that may easily arise when currents are applied to the samples. However, environmental factors such as variations in the system set-up and cell membrane composition, which is likely to vary from cell to cell, can affect the spectral properties of the dye (Hayashi, et al., 1996). Differences in membrane staining and illumination that derive from the staining process and the microscope and camera system set up accounted

for day-to-day variations in the system. Therefore, normalization of the average intensity of the cell membrane at all time-points was used to reduce cell-to cell variation of fluorescence. In addition, no membrane potential changes induced by PBS injections before and after the ATP injection experiment ensured that the cell response was attributed to ATP stimulation, not a result of injection.

Depolarizing responses due to exogenous ATP have been extensively measured electrophysiologically(Grubb and Evans, 1999, Huber-Lang, et al., 1997, Ueno, et al., 1992). In neurons, ATP analogues such as 2-methylthio-ATP, ADP and α,β methylene ATP reported depolarizing responses while neither adenosine nor AMP evoked any response (Ueno, et al., 1992). Normal chondrocytes stimulated by exogenous ATP, UTP 2-methylthioadenosine and α,β-methylene ATP have shown membrane hyperpolarization while no significant response was observed from chondrocytes isolated from osteoarthritis cartilage (Millward-Sadler, et al., 2004). Purinergic receptors in the plasma membrane of cells are excited when bound to extracellular ATP and initiate signaling cascades (Burnstock, 1972, Ralevic and Burnstock, 1998). In chondrocytes, Ca^{2+} signaling through P2 receptors activation was demonstrated by using antagonists of P2 receptors (Elfervig, et al., 2001, Pingguan-Murphy, et al., 2006). In IVD cells, the expression of mRNA for P2X purinergic receptors (P2X$_{4,}$) is consistent with previous studies documenting the involvement of P2X$_4$ receptors in cell membrane depolarization (Hanley, et al., 2004, Kwon, 2012). It was suggested that P2X$_4$ activation permits the influx of Ca^{2+} and Na^+, which in turn depolarizes the cell membrane. Depolarization of the cell membrane opens voltage-dependent calcium channels that enhance Ca^{2+} influx and, as a result, induces ATP

release. This mechanism has been proposed to initiate Ca^{2+} oscillations that drive ATP oscillations during chondrogenesis (Kwon, 2012). In monocyte-derived human macrophages, transient depolarization of the cell membrane was reported to be induced by extracellular ATP possibly via $P2X_4$ receptor activation (Hanley, et al., 2004). In this study, the inhibition of the membrane potential change response to ATP by PPADS (an inhibitor of P2 receptors) also confirmed the involvement of P2 receptor activation, especially $P2X_4$, suggesting that ATP may modulate the biological activities of IVD cells via the P2 purinergic receptors. Although PPADS has been reported to have modest to poor inhibition effects at blocking $P2X_4$ (Soto, et al., 1996), it has also been documented that mouse and human $P2X_4$ receptors displayed more sensitivity to PPADS than the rat $P2X_4$ receptor under the same experimental conditions (Jones, et al., 2000). Moreover, differences in antagonist binding and desensitization were reported between rat and human $P2X_4$ receptors that display 87% sequence homology (Garcia-Guzman, et al., 1997). The human $P2X_4$ receptor showed higher sensitivity to PPADS and more rapid desensitization than the rat $P2X_4$ receptor. Therefore, sensitivity to PPADS may be different among different species.

Since a 3-dimensional agarose gel maintains the original cellular phenotype by resembling the in vivo environment (Gruber, et al., 1997), different responses to ATP observed between the two culture conditions may be due to differences in cell phenotype. In addition, the distinct responses of membrane potential change between AF and NP cells may be due to different embryonic origins. AF cells are derived from the mesenchyme, while NP cells are derived from the notochord. Moreover, AF cells are elongated and

resemble fibroblasts, whereas NP cells are spheroidal and chondrocyte-like in vivo (Buckwalter, 1995).

In this study, the ATP level required to induce membrane potential changes in IVD cells was higher than the reported in electrophysiological recordings in which the EC_{50} of P2 receptors is in the low micromolar range (Soto, et al., 1996). There are two possible reasons for this difference. Firstly, our system detects the potential change of the whole cell membrane while the patch clamp technique can precisely measure transmembrane current induced by activation of a single P2 receptor. Therefore, based on small changes in the florescence intensity of the whole cell membrane triggered by ATP in this study, the major limitation of using a voltage sensitive dye to measure membrane potential changes is its sensitivity compared to precise measurements of the traditional patch clamp technique. Secondly, a high extracellular ATP level (~165 μM (Wang, et al., 2013)) found in the IVD may desensitize the P2 receptors of IVD cells. For instance, previous studies found that normal and osteoarthritic human chondrocytes exhibited different responses to ATP, although there were no differences in expression of P2 receptors between them (Knight, et al., 2009). That observation suggests an ATP signaling desensitization effect due to increased levels of ATP found in osteoarthritic synovial fluid (Knight, et al., 2009, Millward-Sadler, et al., 2004).

CHAPTER 4: THE EFFECTS OF ATP ON THE EXTRACELLULAR MATRIX BIOSYNTHESIS OF IVD CELLS

4.1 INTRODUCTORY REMARKS

The IVD provides the mechanical properties that allow flexion, bending, and torsion of the spine, and transmission of loads through the spinal column. These biomechanical properties are maintained by the composition and organization of the disc's ECM. The interplay of the two ECM main macromolecules, the PG gel and the fibrillar collagen network, determines the mechanical response of the IVD (Roughley, 1976). The IVD cells, which populate the discs at low densities, are responsible for maintaining the proper homeostatic balance of biosynthesis, breakdown, and accumulation of ECM constituents (Ohshima, et al., 1995). These cellular processes determine the disc's mechanical response by maintaining the quality and integrity of the ECM (Buschmann, et al., 1995). In addition, decreasing PG concentration was found with increasing grade of IVD degeneration (Pearce, et al., 1987). In an in vitro study, disc aggrecan (part of the PG family) inhibited nerve growth, which is linked with the development of low back pain (Johnson, et al., 2002). Therefore, it is suggested that detrimental changes in the ECM are associated with IVD degeneration and low back pain.

Maintenance of the ECM is a high energy demanding process that requires glucose and oxygen consumption to produce energy in the form of ATP. Nutrients are supplied mainly by diffusion from blood vessels at the margins of the disc due to the avascular nature of the IVD and transported through the dense ECM to IVD cells (Urban, et al., 2004).

This mechanism of transport may be restricted by factors such as calcification of the endplate or changes in the composition of the ECM (Grunhagen, et al., 2011) which results in detrimental effects on essential cellular activities (e.g., ATP production). A previous study reported that intracellular ATP level declined during the development of spontaneous knee osteoarthritis in guinea pigs, indicating that depletion of ATP is associated with cartilage degeneration (Johnson, et al., 2004). Hence, cellular energy production for the proper synthesis of ECM molecules may be crucial to sustain the integrity and function of the IVD.

During daily activities, the spine is subjected to mechanical forces that influence cell metabolism, gene expression and ECM synthesis in IVD cells (Kasra, et al., 2006, Korecki, et al., 2009, Maclean, et al., 2004, Ohshima, et al., 1995, Walsh and Lotz, 2004). Recent studies have demonstrated that compressive loading promotes ATP production and release in IVD cells in a 3-dimensional agarose gel model (Czamanski, et al., 2011, Fernando, et al., 2011) and in-situ energy metabolism in the IVD (Wang, et al., 2013). Furthermore, high accumulation of extracellular ATP due to the disc's avascular nature was found in the center of young healthy porcine IVD (Wang, et al., 2013). ATP is an extracellular signaling molecule that mediates a variety of cellular activities via purinergic pathways (Burnstock, 1997), including ECM production (Croucher, et al., 2000, Waldman, et al., 2010). Hence, ATP metabolism mediated by compressive loading and extracellular ATP accumulation could be a potential pathway that regulates crucial biological activities in the IVD.

4.2. MATERIALS AND METHODS

Intervertebral disc cells isolation and samples preparation

The IVDs were obtained from mature pigs (~ 250 lbs.) within 2 hours of sacrifice. The NP and AF tissues were harvested and digested in DMEM containing 1 mg/ml type II collagenase, and 0.6 mg/ml protease for 24 hours at 37 °C, 5% CO_2. The cells-enzyme solutions were filtered using a 70μm strainer and cells were isolated by centrifugation. The IVD cells were then re-suspended in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. The cells were mixed at a 1:1 ratio with 4% agarose gel to obtain cell-agarose samples of 1×10^6 cells in 100 μl of 2% agarose. Freshly isolated cells were used since serial passaging was reported to cause phenotypic changes (Chou, et al., 2006). Three-dimensional culture was chosen because of its minimal binding interaction with cells (Knight, et al., 1998, Lee, et al., 2000) and capability to maintain cellular phenotype (Gruber, et al., 1997). All the samples were cultured at 37°C, 5% CO_2 in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic for the duration of the experiments.

PG and collagen content measurements

Samples were cultured for 21 days under different concentrations of ATP (Sigma-Aldrich). Experimental groups included: Control (no ATP), 20 μM and 100 μM ATP treatment groups (NP: n = 9; AF: n = 9 for each group). The cell culture medium was changed three times a week and ATP was administered in each medium change. The duration of the experiment was chosen based on a previous study of chondrocytes

(Croucher, et al., 2000). After 21 days, each sample was lyophilized and then digested overnight in 1 ml of papain at 60^oC. PG content was quantified using the DMMB dye binding assay as previously described in the literature (Farndale, et al., 1986). 150 μl of the samples were further hydrolyzed overnight with 6N hydrochloric acid at 105^oC and assayed for hydroxyproline (HYP) content, as previously described in the literature (Neuman and Logan, 1950). As high HYP content is found in collagen, HYP levels were measured as an indicator of collagen content (Neuman and Logan, 1950). DNA content was quantified in samples digested in papain using a Quant-iT dsDNA HS Assay Kit (Invitrogen Corp.). The PG and collagen levels in each sample were normalized by its DNA content to account for variations in cell number. To evaluate the effects of long-term ATP treatment on PG and collagen contents, each ATP treatment group was normalized by its respective Control group. To evaluate the difference between NP and AF cells, values of the NP cells were normalized by the average of AF Control groups. A one-way ANOVA followed by a post hoc Student Newman Keuls test (SPSS Statistics 20, Chicago, IL) was performed to compare PG and collagen contents between different treatment groups of the same cell type. Student's t-tests were performed to compare PG and collagen levels between NP and AF cells with the same treatment. Significance was taken at $p < 0.05$ in all statistical analysis. Additionally, cell viability was examined using LIVE/DEAD® Cell Viability Assay (Invitrogen Corp.) as instructed by manufacturer.

Gene expression of aggrecan and type II collagen

Samples were cultured for 16 hours with 100 μM ATP (NP: n = 12; AF: n = 9 for Control and ATP treatment group). Total RNA from each sample was obtained using a modified version of the trizol (Tri-Reagent, Molecular Research Center, Cincinnati, OH) protocol. To improve the yield of RNA, 2 ml of trizol were added to the samples to facilitate agarose homogenization. After homogenization, vortexing and incubation for 5 minutes at room temperature, the samples were centrifuged for 10 minutes at 5000 rpm. The supernatants were collected and the trizol protocol was followed starting from the phase separation step. At the end of the procedure, the RNA pellets were left to dry 5 minutes at room temperature and 17 μl of DNase/RNase free water were added. The RNA pellets were left to swell for 5 minutes at room temperature and then stored at -80°C overnight. The following day, the pellets were homogenized and centrifuged at 12000 rpm for 20 minutes at 4°C to collect the supernatant containing the RNA. RNA was quantified using the Qubit RNA BR assay kit (Life Technologies, Carlsbad, CA) and reverse transcribed to cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA), according to the manufacturers' specifications. The levels of mRNA of the anabolic genes aggrecan and type II collagen were measured using real-time RT-PCR (One step Plus, Applied Biosystems) and normalized by that of the endogenous control (18s) and the average of the internal controls. The $2^{-\Delta\Delta C_T}$ method was applied assuming that the amplification efficiencies of the target and the reference genes were approximately equal (Livak and Schmittgen, 2001). Student's t-tests were performed to compare relative changes in gene expression between the Control and the treatment group of the same cell

type. The primer sequences were as follows: aggrecan forward primer: AGACAGTGACCTGGCCTGAC; aggrecan reverse primer: CCAGGGGCAAATGTAAAGG; type II collagen forward primer: TGAGAGGTCTTCCTGGCAAA; type II collagen reverse primer: ATCACCTGGTTTCCCACCTT; 18S forward primer: CGGCTACCACATCCAAGGA; 18S reverse primer: AGCTGGAATTACCGCGGCT. The sizes of PCR products for aggrecan, type II collagen and 18S were 151, 161 and 188 bp, respectively.

Intracellular ATP measurements

Samples were cultured for 2 hours with 100 μM ATP (NP: n = 9; AF: n = 9 for Control and ATP treatment group) and then dissolved in lysis buffer consisting of 15% 1.5 M NaCl, 15% 50 mM EDTA, 1% Triton-X 100 and 10% 100 mM Tris–Cl at pH 7.4 by heating at 65 °C. The lysates were centrifuged for 10 minutes at 9000 rpm and the supernatants were collected for intracellular ATP and DNA content measurements. Intracellular ATP was measured using the Luciferin-luciferase method (Sigma-Aldrich) and a plate reader (DTX880, Beckman Coulter, Brea, CA). Values of intracellular ATP were quantified and normalized by DNA content. To compare variations of intracellular ATP on each cell type, a student t-test was performed between the Control and the treatment group. To evaluate differences between NP and AF cells, values of NP cells were normalized by the average of AF Control groups and student t-tests were performed.

Inhibition of P2 receptors

The samples were treated with the inhibitor of P2 receptors, PPADS. Briefly, the samples were incubated for 30 minutes with 30 μM of PPADS (Millward-Sadler, et al., 2004). Then, ATP was added to the culture medium to obtain a final concentration of 100 μM. Gene expression was analyzed after 16 hours of the addition of ATP (NP: n = 3, AF: n = 4 for Control and inhibitor groups).

4.3 RESULTS

The effects of ATP treatment on the accumulation of PG and collagen

Cell viability staining confirmed that there were no detrimental effects on cells after 21 days of 100 μM ATP treatment (Figure 17). In NP cells, 100 μM ATP treatment significantly increased PG and collagen as compared to both the 20 μM ATP and Control groups after 21 days of treatment (Figure 18A and Figure 19A). No significant difference was found in ECM deposition between the Control and the 20 μM ATP groups. In AF cells, both ATP treatment groups exhibited significantly higher PG and collagen levels than the Control group, while the 100 μM ATP group showed a significant higher increase in the contents of PG and collagen than the 20 μM ATP group after 21 days of treatment (Figure 18B and Figure 19B). The PG content deposited by NP cells was significantly higher than AF cells under all treatment conditions (Figure 20A). The collagen content accumulated by NP cells was significantly higher in the Control and the 100 μM ATP groups compared to their respective AF group. No significant difference in collagen content was found between NP and AF cells treated with 20 μM ATP (Figure 20B).
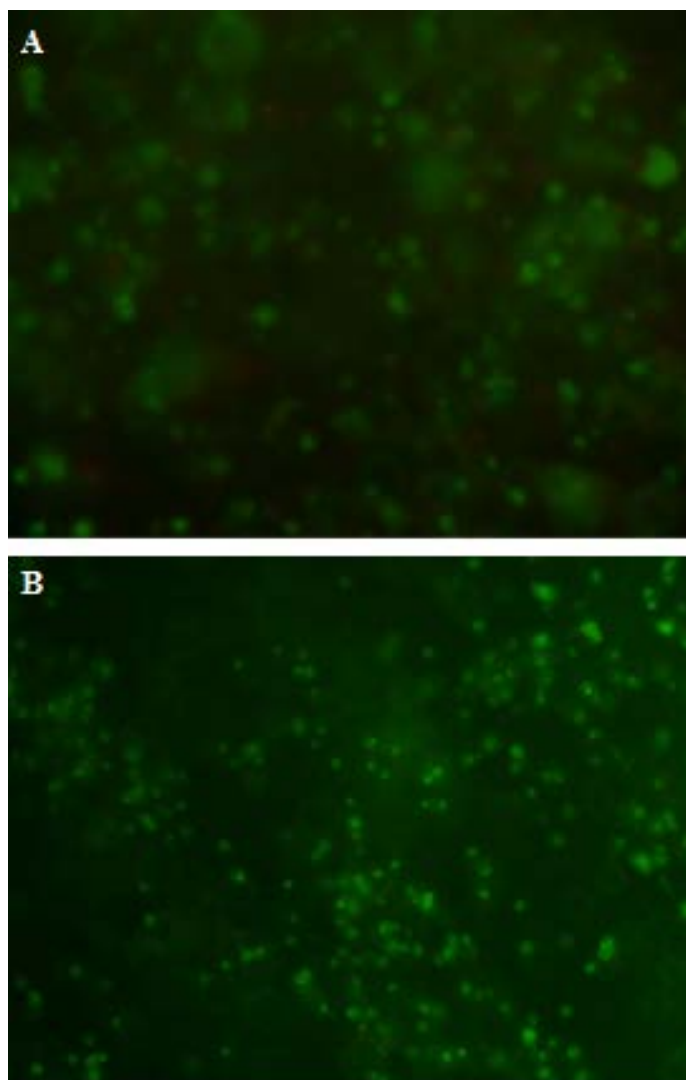
Figure 17. Viability of IVD cells treated with 100 μM ATP for 21 days (green/red: alive/dead cells). A. NP cells. B. AF cells
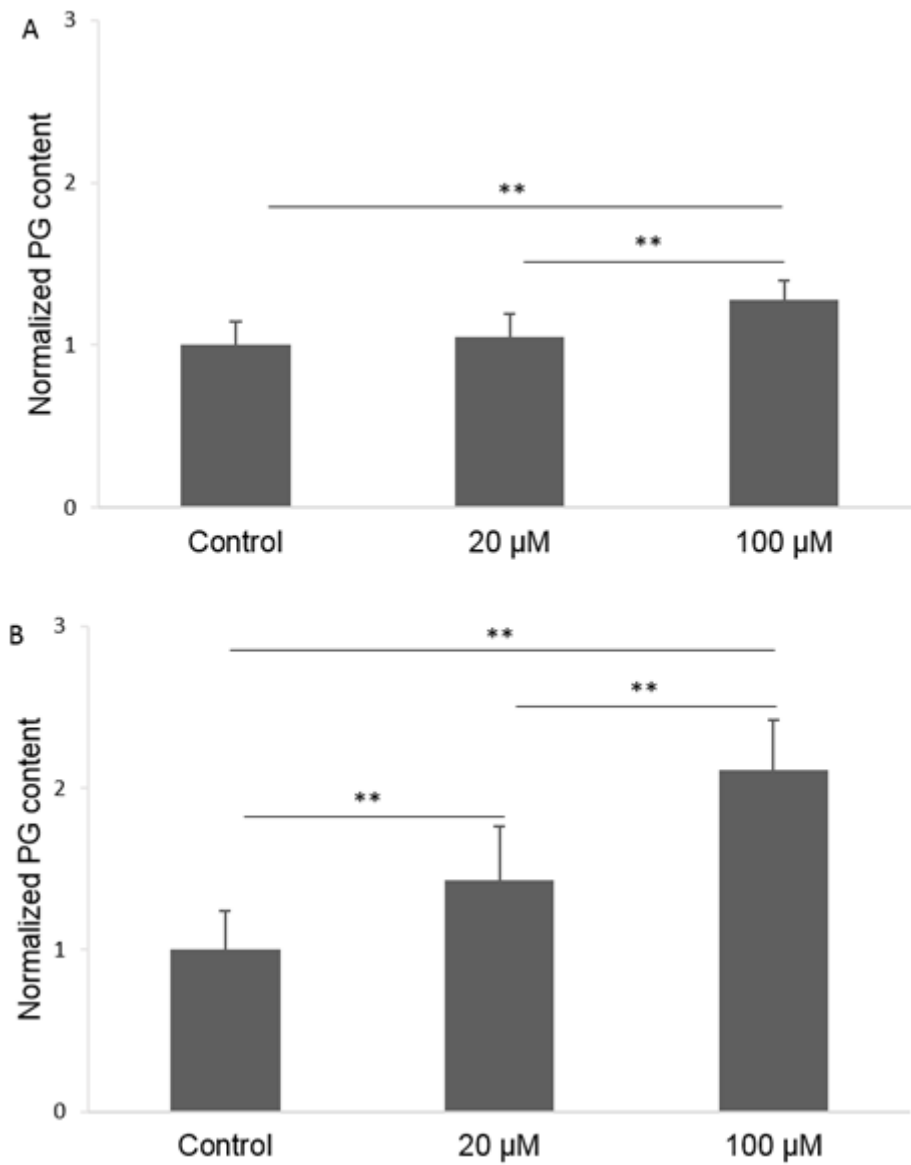
Figure 18. Proteoglycan content of IVD cells treated with ATP at different concentrations for 21 days. A. NP cells. B. AF cells (n = 9; **p < 0.01 indicates statistically significant differences between groups)
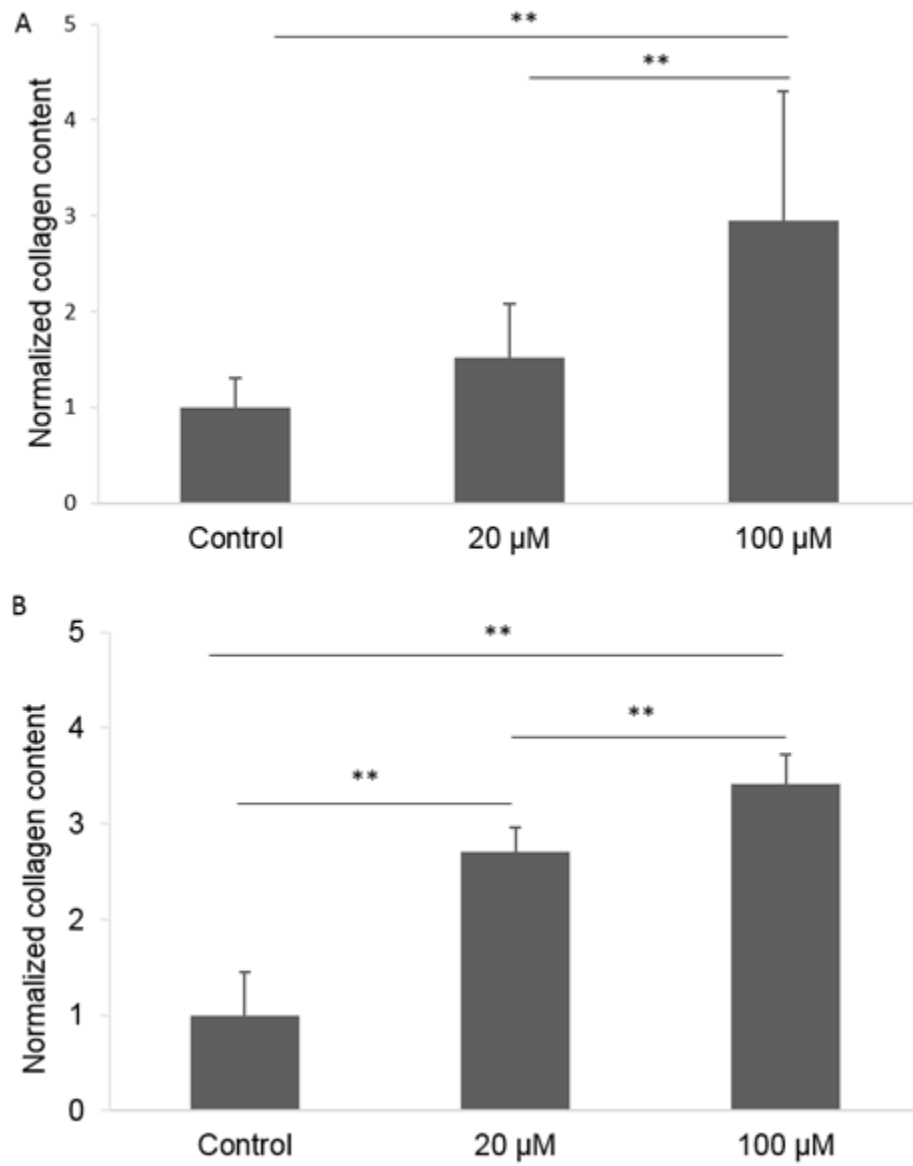
Figure 19. Collagen content of IVD cells treated with ATP at different concentrations for 21 days. A. NP cells. B. AF cells. (n = 9; **p < 0.01 indicates statistically significant differences between groups)
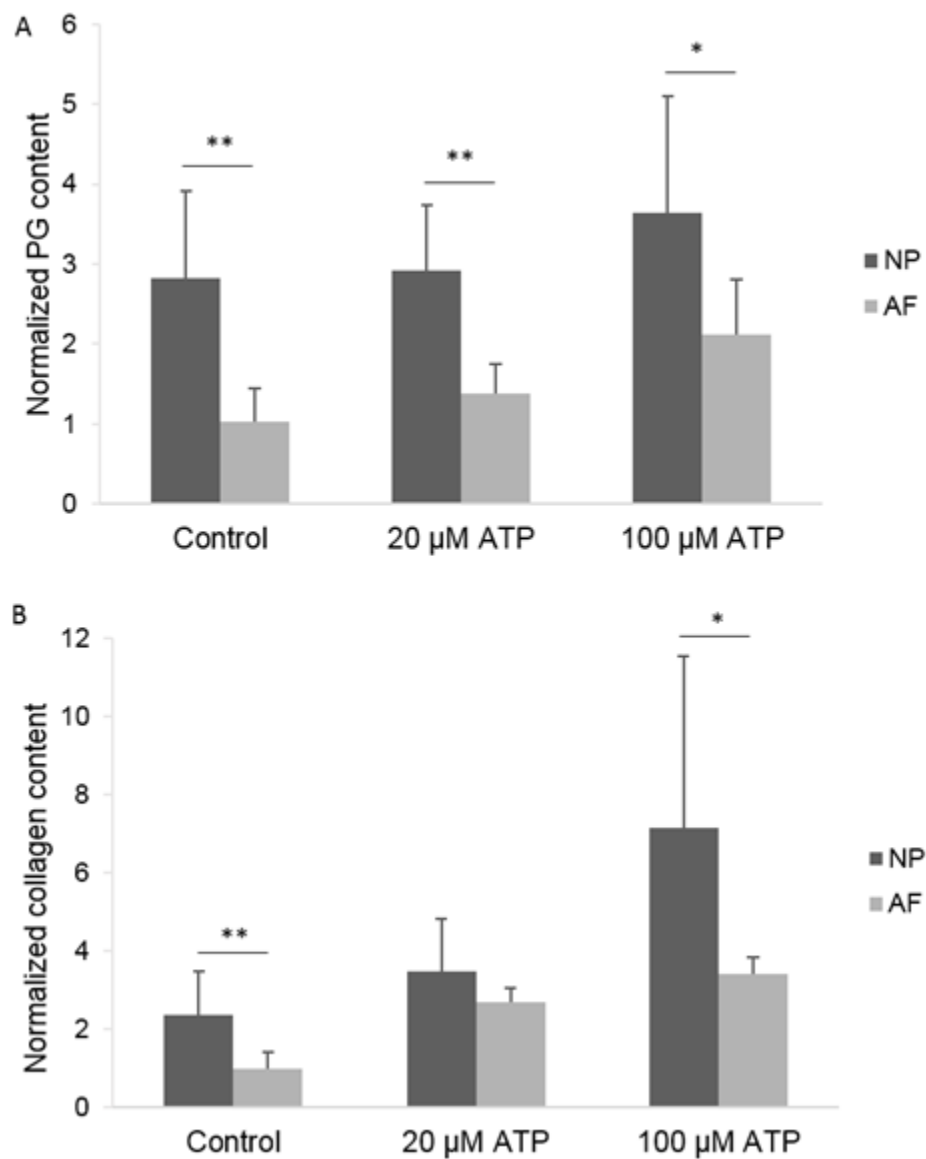
Figure 20. Comparison of ECM main macromolecules contents between NP and AF cells treated with ATP at different concentrations for 21 days. NP values were normalized by the average of the Control groups of AF cells. A. PG content. B. Collagen content. (n = 9; *p < 0.05 and **p < 0.01 indicate statistically significant differences between groups)

The effects of ATP treatment on the gene expression of aggrecan and type II collagen

The gene expressions of aggrecan and type II collagen in NP and AF cells were significantly higher after 16 hours of 100 µM ATP treatment compared to control conditions (Figure 21).

The effects of ATP treatment on the intracellular ATP content

Intracellular ATP content significantly increased in NP and AF cells after 2 hours of 100 µM ATP treatment compared to their Control groups (Figure 22A). In addition, a comparison between NP and AF cells showed that NP cells have a significant higher intracellular ATP content than AF cells under all conditions (Figure 22B).
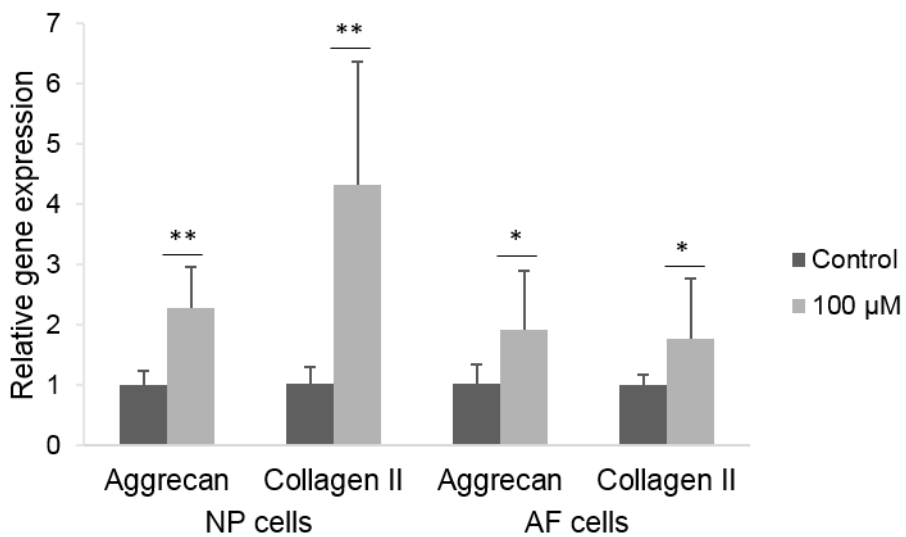
Figure 21. Aggrecan and type II collagen gene expressions of NP and AF cells treated with 100 μM ATP for 16 hours. (n = 12 for NP, n = 9 for AF; *p < 0.05 and **p < 0.01 indicate statistically significant differences between groups)
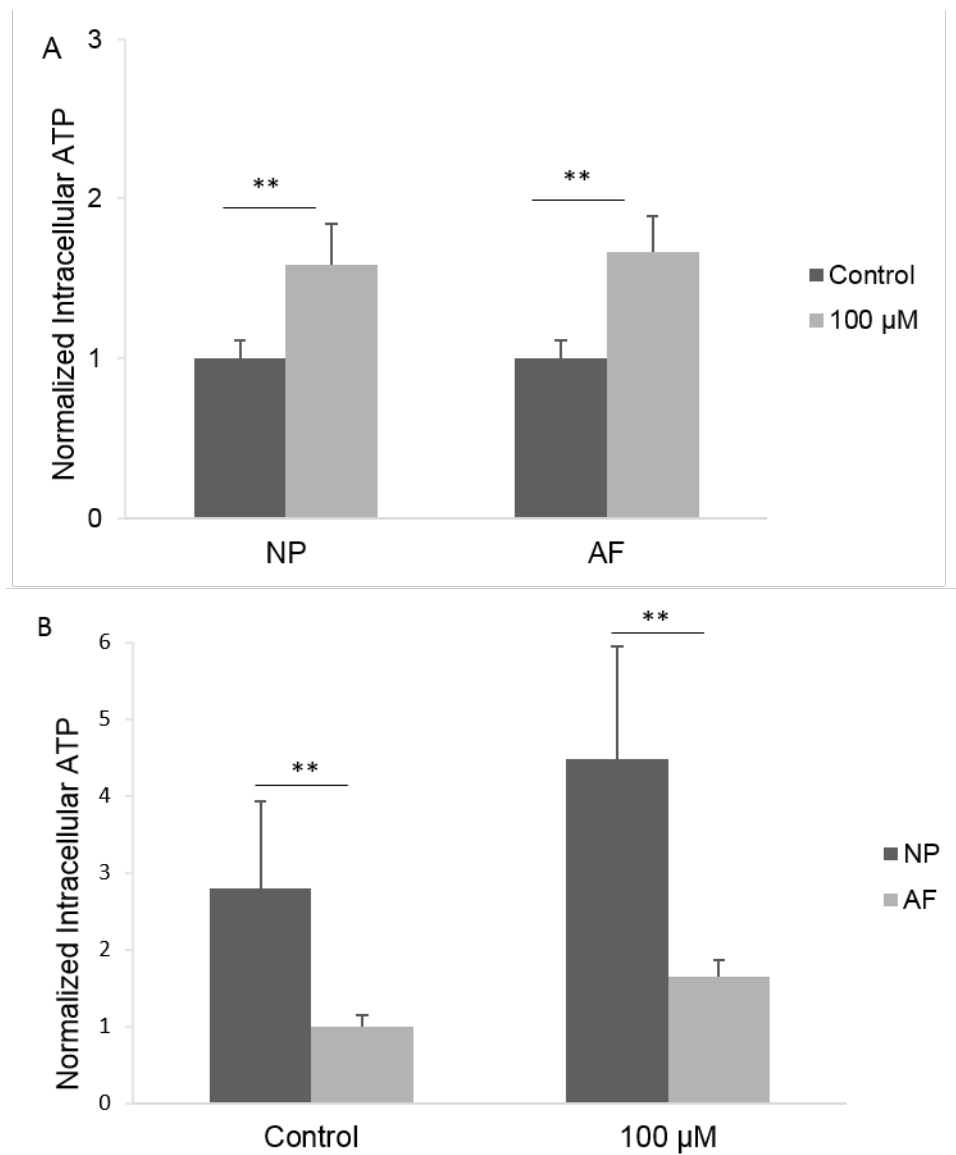
Figure 22. Intracellular ATP content of IVD cells treated with 100 μM ATP for 2 hours.

A. Comparison between experimental groups of the same cell type. B. Comparison between NP and AF cells. (n = 9; **p < 0.01 indicates statistically significant differences between groups)

Inhibition of P2 receptors

PPADS was used to examine whether the purinergic pathway has a role in the ECM biosynthesis of IVD cells. No significant difference in gene expression between the Control and the PPADS groups was found in NP or AF cells.

4.4 DISCUSSION

Increased matrix breakdown, altered matrix synthesis (reduced synthesis of aggrecan and synthesis of type I collagen instead of type II collagen) and apoptosis are among the metabolic changes that contribute to IVD degeneration (Adams and Roughley, 1976, Freemont, 2009). Moreover, aggrecan has shown to inhibit nerve growth in vitro, suggesting that loss of aggrecan is associated with ingrowth of nerves that may cause low back pain in degenerated IVDs (Johnson, et al., 2002). Proper biosynthesis of ECM in the IVD is a complex process that requires an extensive amount of ATP to be accomplished, especially PG biosynthesis which uses ATP as an energy source and building block (Hirschberg, et al., 1998). The finding that high levels of extracellular ATP promoted ECM biosynthesis and intracellular ATP production in IVD cells suggests that the high accumulation of extracellular ATP found in the NP (Wang, et al., 2013) may play an important role in maintaining a healthy ECM structure of the IVD. Furthermore, to our knowledge, this is the first study to demonstrate that extracellular ATP influences ECM biosynthesis and intracellular ATP content in IVD cells.

In skin cells, galactosyltransferase-I, an enzyme that synthesizes the linkage region between the core protein and the glycosaminoglycan chains of PGs, enhanced its activity after incubation with ATP (Higuchi, et al., 2001). In addition, chondrocytes cultured with

ATP demonstrated to increase proteoglycan and collagen deposition (Croucher, et al., 2000, Waldman, et al., 2010). Those previous studies support this study's finding, suggesting that extracellular ATP can mediate cellular ECM biosynthesis. In addition, it was also found that upregulation of ECM synthesis by exogenous ATP was diminished by antagonist of P2 receptors, suggesting the involvement of a purinergic signaling pathway (Waldman, et al., 2010).

This study found that a lower ATP concentration (i.e., 20 μM) induced a significant increase in accumulation of both ECM molecules by AF cells compared to NP cells. This finding suggests that NP cells may be less sensitive to extracellular ATP than AF cells. The different cellular responses to ATP between NP and AF cells could be explained a previous study which found that NP cells reside in an environment with a higher level of extracellular ATP (~165 μM) than AF cells (<10 μM) (Wang, et al., 2013). Furthermore, the findings of higher PG and collagen accumulations and intracellular ATP content by the NP groups compared to the AF counterpart groups are consistent with previous studies, which suggested that NP cells are more metabolically active than AF cells (Czamanski, et al., 2011, Fernando, et al., 2011). The differences in the metabolic activities between AF and NP cells may be explained by differences in cell phenotypes in which AF cells are elongated and resemble fibroblasts, whereas NP cells are spheroidal and chondrocyte-like (Buckwalter, 1995). Also, both cell types have distinct embryonic origins. NP cells are derived from the notochord and AF cells are derived from the mesenchyme (Roughley, 1976).

The IVD is subjected to static and dynamic loading at different magnitudes and frequencies during daily activities. Mechanical loading activates different mechanotransduction pathways, which can lead to modification of cell function, metabolism and gene expression (Chowdhury and Knight, 2006, Maclean, et al., 2004). Previous studies showed that mechanical loading mediates ECM biosynthesis of IVD cells (Kasra, et al., 2006, Korecki, et al., 2009, Maclean, et al., 2004, Ohshima, et al., 1995, Walsh and Lotz, 2004). It was also found that mRNA expressions of aggrecan and collagens in NP and AF regions were altered by specific mechanical loading regimens (Hutton, et al., 1999, Maclean, et al., 2004, Neidlinger-Wilke, et al., 2006), while similar effects of mechanical of loading were observed on the incorporation of $[^{35}S]$-sulfate and $[^{3}H]$-proline (measures of protein synthesis) into collagens and PGs, respectively (Hutton, et al., 1999). In this study, mRNA levels of aggrecan and type II collagen were up-regulated by extracellular ATP, which also correlated with their corresponding protein synthesis. Furthermore, inhibition of gene expression by PPADS demonstrates that the ATP pathway is involved in the ECM biosynthesis of IVD cells. Since previous studies have shown that static and dynamic loading alter ATP production and release in IVD cells (Czamanski, et al., 2011, Fernando, et al., 2011) and in-situ energy metabolism in the IVD (Wang, et al., 2013), the findings of this study suggest that mechanical loading may affect ECM production of IVD cells via extracellular ATP pathway.

In this study, it was also found that extracellular ATP treatment promoted intracellular ATP production in IVD cells. This finding is consistent with previous studies which reported that treatment with extracellular nucleotides or adenosine increased the

concentration of intracellular ATP (Andreoli, et al., 1990, Lasso de la Vega, et al., 1994). In cancer cells, the action of exogenous ATP appeared to be mediated by the hydrolysis of extracellular ATP and subsequently uptake of adenosine into cells, which increased intracellular ATP contents (Lasso de la Vega, et al., 1994). In human umbilical vein endothelial cells, treatment with 25 μM of ATP, ADP, AMP or adenosine significantly raised intracellular ATP levels through the same mechanism (i.e, adenosine uptake) (Andreoli, et al., 1990). In addition, previous studies have also reported that extracellular ATP signaling via P2X$_4$ receptor mediates intracellular ATP oscillations, which are involved in prechondrogenic condensation in chondrogenesis (Kwon, 2012, Kwon, et al., 2012). Hence, these evidence suggest that intracellular ATP production could be mediated by hydrolisis of extracellular ATP, subsequent uptake of adenosine into cells, and/or activation of purinergic receptors on the cell membrane.

Due to the avascular nature of the IVD, delivery of nutrients to IVD cells relies on diffusion. In humans, about 25% of water is extruded from the disc due to high loads during daily activities (Paesold, et al.). A decrease in disc hydration reduces supply (diffusion) of oxygen and glucose for cellular ATP production, which is essential for maintaining cell viability and normal ECM production especially in the center of the disc (i.e., NP region). Since mechanical loading could promote hydrolysis of extracellular ATP which is highly accumulated in the NP (Wang, et al., 2013), intracellular ATP levels in IVD cells could be increased via the adenosine uptake mechanism described in the previous section, compensating the effects of mechanical loading on nutrient supply. When disc hydration is recovered during rest at night (Boos, et al., 1993), cells could produce more ATP which

could be released and accummulated in the ECM. Therefore, high accumulation of extracellular ATP in the NP region (Wang, et al., 2013) could play an important role in maintaining normal activities of IVD cells. It also suggests a mechanobiological pathway for regulating ECM biosynthesis via ATP metabolism (Figure 23).
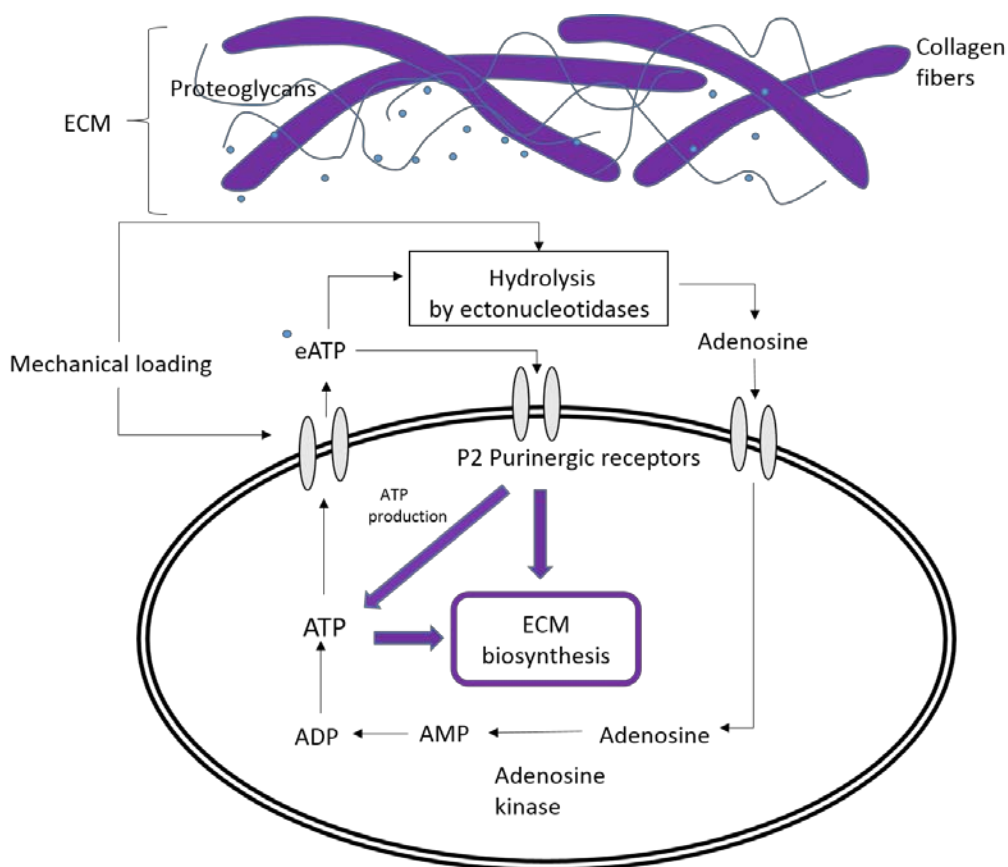
Figure 23. A postulated mechanobiological pathway that regulates EMC biosynthesis in IVD cells via ATP metabolism. Mechanical loading stimulates ATP release (Czamanski, et al., 2011, Fernando, et al., 2011) via a transport mechanism, through a membrane channel, or by leakage through a damaged cellular membrane (Graff, et al., 2000). Extracellular ATP (eATP) activates P2 purinergic receptors on the cell membrane, which are involved in the ECM biosynthesis process (Chowdhury and Knight, 2006) and also in the production of ATP (Kwon, 2012, Kwon, et al., 2012). Mechanical loading may promote eATP hydrolysis (Wang, et al., 2013). Adenosine, which results from the hydrolysis of eATP, is uptaken into the cell and adenosine kinase rephosphorylates adenosine to AMP

that is subsequently rephosphorylated into ATP (Andreoli, et al., 1990, Lasso de la Vega,

et al., 1994), which serves as an energy source and building block for ECM biosynthesis

CHAPTER 5: THE EFFECTS OF ADENOSINE ON THE EXTRACELLULAR MATRIX BIOSYNTHESIS OF IVD CELLS

5.1 INTRODUCTORY REMARKS

In order to sustain proper synthesis of ECM molecules, IVD cells obtain nutrients such as glucose and oxygen from blood vessels at the margins of the disc (Urban, et al., 2004). Due to the avascular nature of the IVD, nutrients are mainly transported by diffusion through the dense ECM (Urban, et al., 2004). IVD cells consume those nutrients to produce energy in the form of ATP for essential cellular activities, including the high energy demanding process of ECM synthesis. However, calcification of the endplate or changes in the ECM structure may hinder diffusion of nutrients and substrates (Grunhagen, et al., 2011), affecting cellular activities such as ATP production. Hence, it is suggested that loss of nutrient supply causes improper ECM maintenance and loss of cells, leading to disc degeneration (Urban, et al., 2004).

The spine is subjected to mechanical forces that influence metabolism, ECM synthesis and gene expression in the IVD (Handa, et al., Illien-Junger, et al., 1976, Ishihara, et al., 1996, Maclean, et al., 2004, MacLean, et al., 2003, Ohshima, et al., 1995, Walsh and Lotz, 2004, Wang, et al., 2013). Previous studies have demonstrated that static and dynamic compressions promote ATP production and release in IVD cells (Czamanski, et al., 2011, Fernando, et al., 2011) and energy metabolism in the IVD (Wang, et al., 2013). Additionally, a recent study reported high accumulation of extracellular ATP in the NP of young healthy porcine IVD ($\sim 165\ \mu M$) (Wang, et al., 2013).

Furthermore, the previously mentioned study found that compressive loading reduced extracellular ATP content in the NP, suggesting that compressive loading may promote ATP hydrolysis (Wang, et al., 2013). ATP hydrolysis may increase extracellular accumulation of adenine derivatives such as adenosine due to its avascular nature. Since adenosine has demonstrated its ability to maintain ECM homeostasis (Tesch, et al., 2004) and to regulate different cellular activities by delivering signals via P1 purinergic receptors (Jacobson and Gao, 2006, Ramkumar, et al., 2001), hydrolysis of ATP promoted by mechanical stimulation may subsequently increase accumulation of adenosine which may influence cellular activities in the IVD via P1 purinergic receptors.

5.2 MATERIALS AND METHODS

Intervertebral disc cells isolation and samples preparation

IVD tissues were harvested as described in Chapter 4. After tissue digestion and cells isolation, cell-agarose samples of 1×10^6 cells were obtained by mixing IVD cells at a 1:1 ratio with 4% agarose gel (final volume: 100 μl). In order to prevent phenotypic changes caused by serial passaging, freshly isolated cells were used in all the experiments (Chou, et al., 2006). In addition, three-dimensional culture provided an environment able to maintain cellular phenotype (Gruber, et al., 1997). All the samples were culture at 37°C, 5% CO_2 in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic for the duration of the experiments.

PG and collagen content measurements

The experimental groups of samples were cultured under different concentrations of adenosine (Sigma-Aldrich) for 21 days (Control, 20 µM and 100 µM adenosine treatment groups; NP: n = 9; AF: n = 9 for each group). Adenosine was administered three times a week in each medium change. At the end of the experiment, the samples were lyophilized and then digested in 1 ml of papain at 60ºC. The DMMB dye binding assay was used to quantify PG content and the HYP assay was used to quantify collagen content in each sample, as described in Chapter 4. To account for variations in cell number, measurements of each assay were normalized by DNA content. To evaluate PG and collagen accumulations of the same cell type, each adenosine treatment group was normalized by its respective Control group. To evaluate the difference between NP and AF cells, values of the NP cells were normalized by the average of AF Control groups. Comparisons of PG and collagen contents between different experimental groups of the same cell type were performed by one-way ANOVA followed by post hoc Student Newman Keuls test (SPSS Statistics 20, Chicago, IL). Comparisons of PG and collagen levels between NP and AF cells of the same group of treatment were performed by student's t-tests. Significance was taken at $p < 0.05$ in all statistical analysis.

Gene expression of aggrecan, type II collagen and purinergic receptors

The groups of samples were cultured with 100 µM adenosine for 16 hours (Control and 100 µM adenosine treatment groups; NP: n = 9; AF: n = 9 for each group). The trizol protocol was used to obtain total RNA from the samples and the RNA was transcribed to

cDNA, as described in Chapter 4. Real-time RT-PCR (One step Plus, Applied Biosystems) was performed to measure levels of mRNA of aggrecan and type II collagen genes. The results were normalized by that of the endogenous control (18s) and the average of the internal controls and the $2^{-\Delta\Delta C_T}$ method was applied. Comparisons of the relative change in gene expression between the Control and the treatment group of the same cell type were performed by student's t-tests. The mRNA expression of P1 purinergic receptors of IVD cells seeded in agarose gel was examined by electrophoresing the PCR products on a 2% ethidium bromide-stained agarose gel. The primer sequences of the P1 receptors were as follow: A1 forward primer: ACACACGGCAACTCGGCCATG; A1 reverse primer: CGGGGAGATAAGGGAGACAAGTGGG; A2A forward primer: CTACCGCATCCGTGAGTTCCGC; A2A reverse primer: ACCACATTCCCTCACACTCCCTCC; A2B forward primer: CAAGTCACTGGCTATGATTGT; A2B reverse primer: ACTTGGGCTTCTCCTTCGCCC; A3 forward primer: TGTCATCCGGCACAAACTCAATCA; A3 reverse primer: TGGGACAGCAGGATGCCCAAGT. The sizes of PCR products for A1, A2A, A2B and A3 were 245, 259, 173 and 210 bp, respectively.

Intracellular ATP measurements

The groups of samples were cultured with 100 μM adenosine for 2 hours (Control and 100 μM adenosine treatment groups; NP: n = 9; AF: n = 9 for each group). Intracellular ATP was measured using the Luciferin-luciferase method, as described in Chapter 4. The results were normalized by DNA content in each sample. Comparisons of the intracellular ATP content between the Control and the treatment group of the same cell type were performed by student's t-tests. To evaluate differences between NP and AF cells, values of NP cells were normalized by the average of AF Control groups and student t-tests were performed.

Inhibition of P1 receptors

The samples were treated with the following inhibitors of P1 receptors: 0. 1 μM of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; A1 receptor antagonist; Sigma-Aldrich); 10 μM of 8-(3-Chlorostyryl)caffeine (CSC; A2A receptor antagonist; Tocris Bioscience, Ellisville, MO) and 0. 1 μM of N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazolinyl]-urea (VUF 5574; A3 receptor antagonist, Sigma-Aldrich) (Hinze, et al., 2012, Pugliese, et al., 2006, Sakakibara, et al., 2010). Briefly, the samples were incubated with P1 receptors inhibitors for 30 minutes . Then, adenosine was added to the culture medium to obtain a final concentration of 100 μM. Gene expression was analyzed after 16 hours of the addition of adenosine (NP and AF: n = 4 for Control and each adenosine inhibitor groups).

5.3 RESULTS

The effects of adenosine treatment on the accumulation of PG and collagen

In NP cells, the adenosine treatment groups exhibited significantly higher PG and collagen levels than the Control group, while the 100 μM adenosine group showed a significant higher increase in the contents of PG and collagen than the 20 μM adenosine group after 21 days of treatment (Figure 24A and Figure 25A). In AF cells, the 100 μM adenosine treatment significantly increased PG content than the 20 μM adenosine and Control groups after 21 days of treatment. No significant difference in PG was found between the Control and the 20 μM adenosine groups (Figure 24B). Moreover, the adenosine treatment groups showed significantly higher collagen levels than the Control group while the 100 μM group showed a significant higher increase in collagen content than the 20 μM adenosine group after 21 days of treatment (Figure 25B). A comparison between NP and AF cells showed that the PG and collagen contents accumulated by NP cells were significantly higher than their AF counterpart under all conditions (Figure 26).
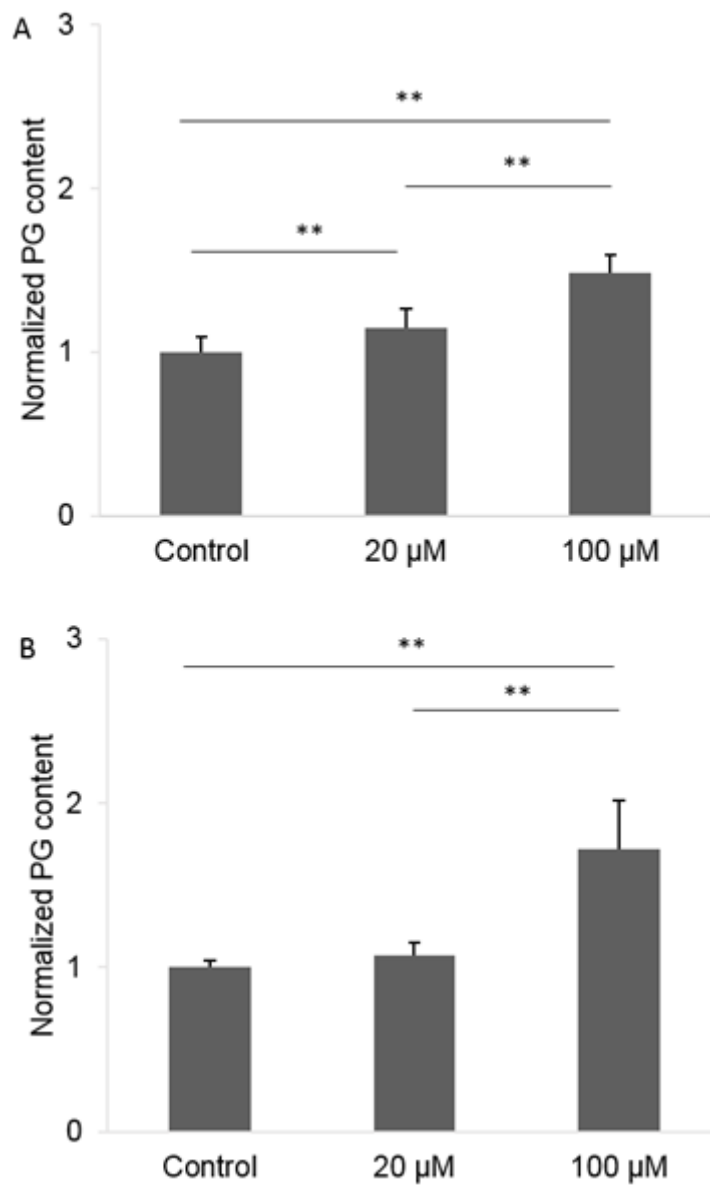
Figure 24. Proteoglycan content accumulated by IVD cells treated with adenosine at different concentrations for 21 days. A. NP cells. B. AF cells (n = 9; **$p < 0.01$ indicates statistically significant differences between groups)
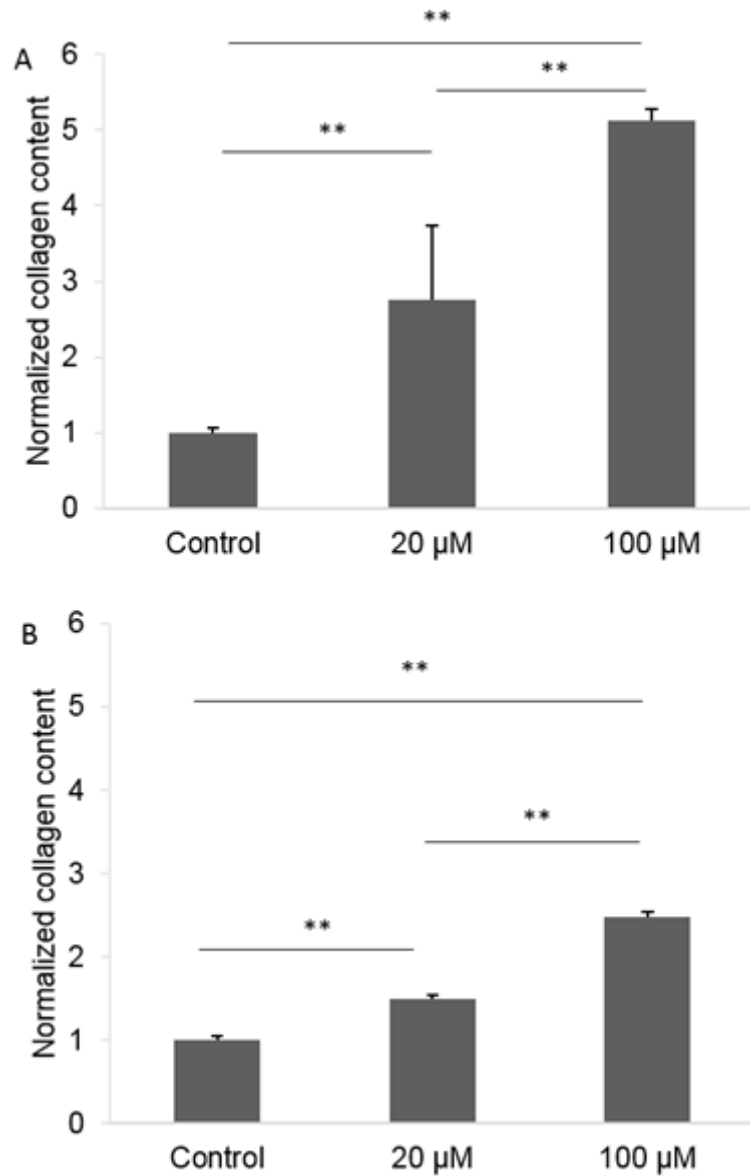
Figure 25. Collagen content accumulated by IVD cells treated with adenosine at different concentrations for 21 days. A. NP cells. B. AF cells. (n = 9; **p < 0.01 indicates statistically significant differences between groups)
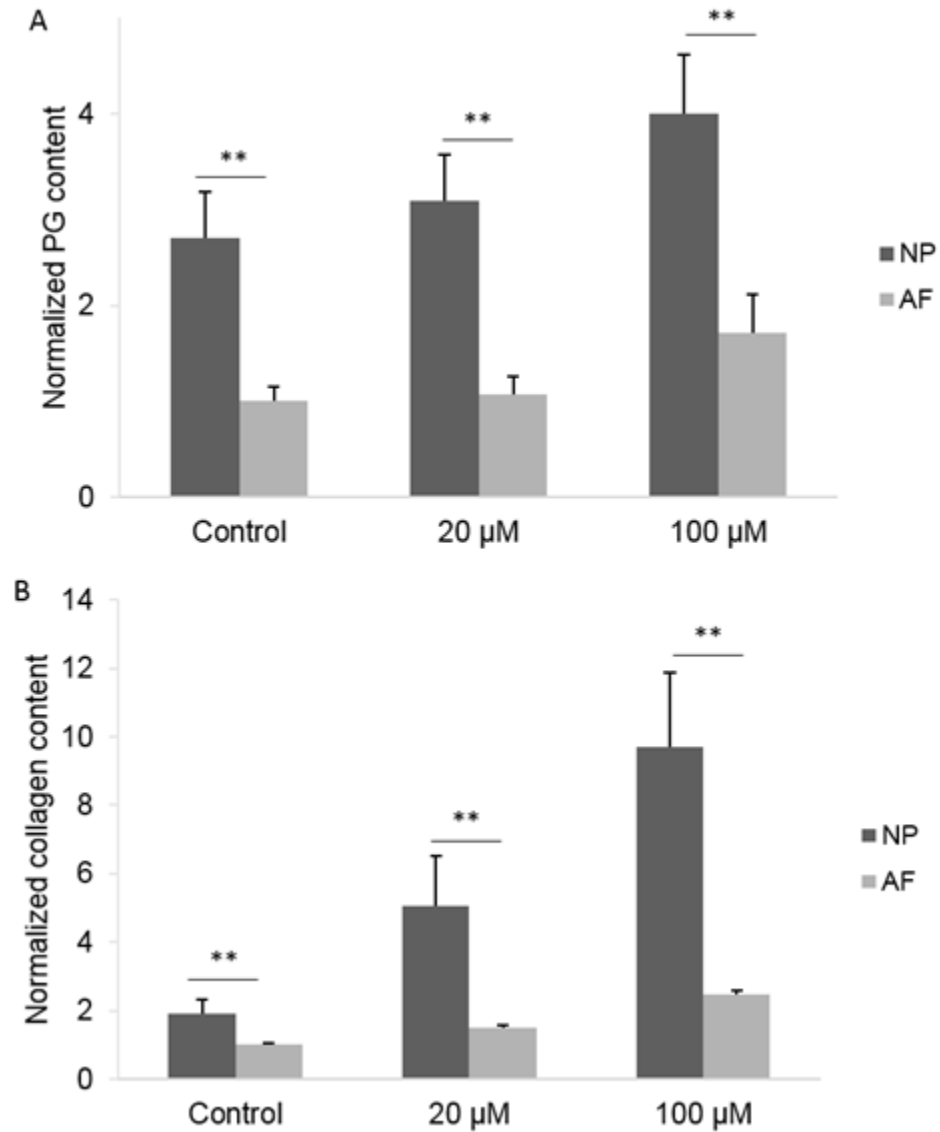
Figure 26. Comparison of ECM main macromolecules contents between NP and AF cells treated with adenosine at different concentrations for 21 days. NP values were normalized by the average of the Control groups of AF cells. A. PG content. B. Collagen content. (n = 9; **p < 0.01 indicates statistically significant differences between groups)

The effects of adenosine treatment on the gene expression of aggrecan and type II collagen

NP and AF cells expressed a significant high relative gene expression of aggrecan and type II collagen after 16 hours of 100 μM adenosine treatment compared to control conditions (Figure 27).
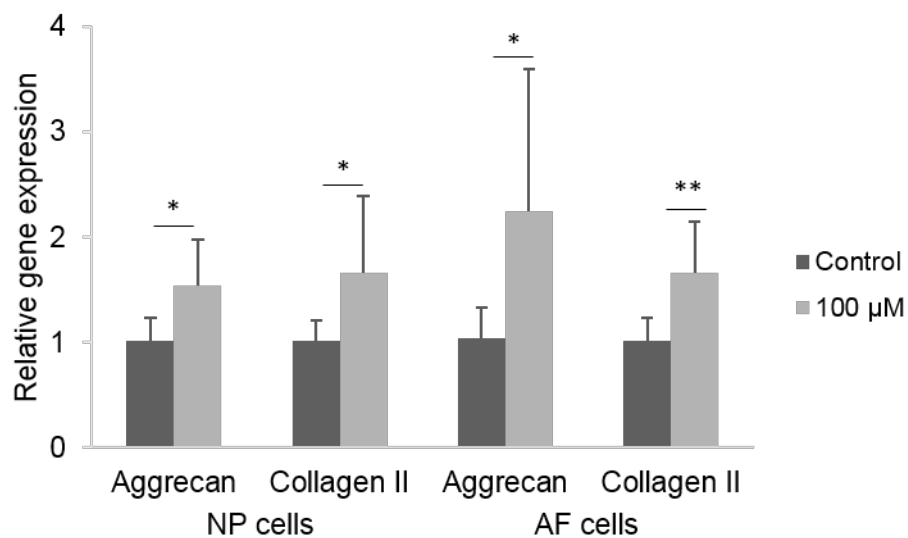
Figure 27. Aggrecan and type II collagen gene expressions of NP and AF cells treated with 100 μM adenosine for 16 hours. (n = 9 for NP, n = 9 for AF; *p < 0.05 and **p < 0.01 indicate statistically significant differences between groups)

Gene expression of purinergic receptors

The genes of the A1, A2A and A3 purinergic receptors were expressed by AF and NP cells while A2B receptor was not expressed in either cell type (Figure 28).
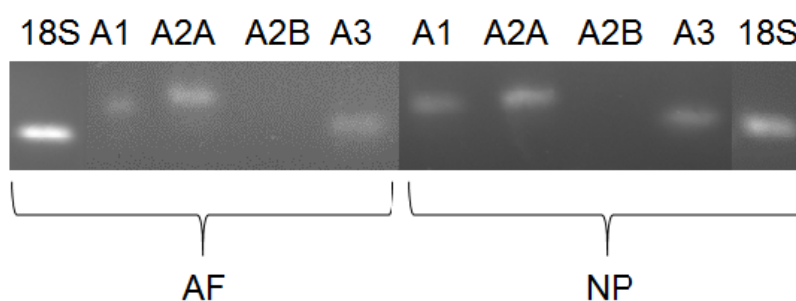
Figure 28. Gene expression of P1 purinergic receptors in NP and AF cells cultured in 3-dimensional agarose gel.

The effects of adenosine treatment on the intracellular ATP content

The intracellular ATP content significantly increased in NP and AF cells after 2 hours of 100 μM adenosine treatment compared to their Control groups (Figure 29A). A comparison between NP and AF cells showed that NP cells have a significant higher intracellular ATP content than AF cells under all conditions (Figure 29B).
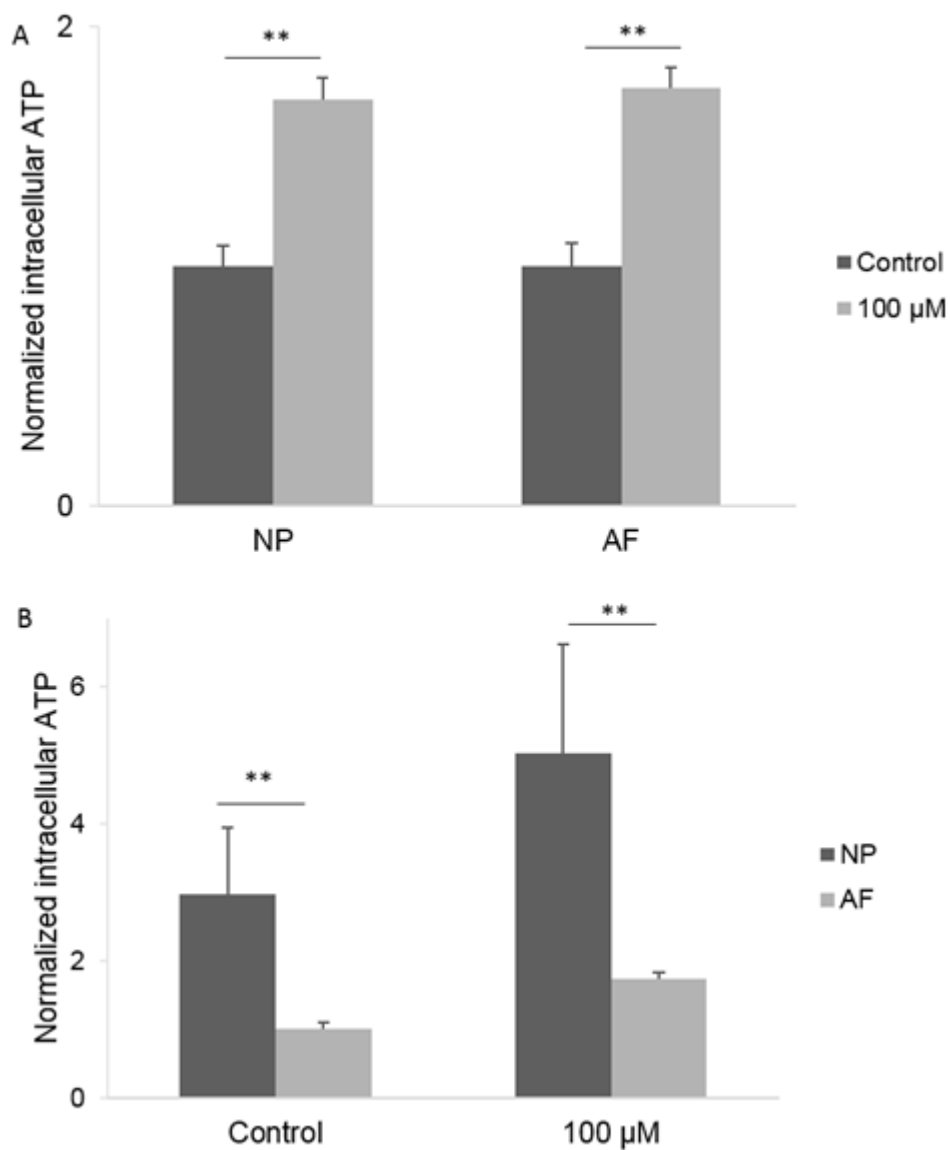
Figure 29. Intracellular ATP content of IVD cells treated with 100 μM adenosine for 2 hours. A. Comparison between experimental groups of the same cell type. B. Comparison between NP and AF cells. (n = 9; **p < 0.01 indicates statistically significant differences between groups)

Inhibition of P1 receptors

DPCPX, CSC and VUF 5574 were used to examine whether A1, A2A or A3 adenosine receptors are involved in the ECM biosynthesis of IVD cells, respectively. In NP cells, no significant difference in gene expression was found between the Control and the inhibitors groups. Moreover, only CSC blocked up-regulation of gene expression in adenosine-treated AF cells.

5.4 DISCUSSION

Several detrimental changes accompany IVD degeneration, including increased breakdown of the matrix, decreased synthesis of aggrecan and increased synthesis of type I collagen instead of type II collagen and cell death (Freemont, 2009). It is suggested that loss of PGs is a factor preceding disc degeneration since low PG concentration has been reported in degenerated IVDs (Pearce, et al., 1987). In addition, disc aggrecan was reported to inhibit nerve growth in vitro, indicating that maintenance of a proper ECM is an important factor for preventing disc innervation that may cause low back pain (Johnson, et al., 2002). The finding that adenosine treatment increased PG and collagen synthesis and also intracellular ATP production in IVD cells suggests that the ATP hydrolysis promoted by mechanical loading in the NP (Wang, et al., 2013) results in increased levels of extracellular adenine derivatives (i.e. adenosine) that influences the ECM biosynthesis in the IVD. Furthermore, this is the first study reporting the effects of adenosine on ECM biosynthesis and intracellular ATP production in IVD cells and expression of adenosine receptors in IVD cells.

In a previous study, cartilage matrix degradation (degradation of PG and higher levels of MMP-3 and MMP-13) was promoted by depletion of endogenous adenosine through incubation with adenosine deaminase, which depleted extracellular adenosine (Tesch, et al., 2004). In this study, adenosine treatment for 21 days induced a significant increase in accumulation of PG and collagen by IVD cells. Moreover, higher accumulation of both ECM components and higher intracellular ATP content in the NP groups compared to their respective AF groups suggest that NP cells are metabolically more active than AF cells, which was reported in previous studies (Czamanski, et al., 2011, Fernando, et al., 2011). This difference in metabolic activities may be explained by their distinct embryonic origins and phenotypes (Buckwalter, 1995, Roughley, 2004).

Previous studies have demonstrated that the IVD is sensitive to mechanical stimulation. In studies of the whole IVD and 3-dimensional culture of IVD cells, mechanical loading has shown to influence ECM biosynthesis (Kasra, et al., 2006, Korecki, et al., 2009, Maclean, et al., 2004, Ohshima, et al., 1995, Walsh and Lotz, 2004) while specific loading regimens have reported to alter the gene expression of aggrecan and collagens (Hutton, et al., 1999, Maclean, et al., 2004, Neidlinger-Wilke, et al., 2006). Furthermore, changes in aggrecan and type II collagen gene expression correlated with their corresponding protein synthesis in IVD cells subjected to hydrostatic pressure (Hutton, et al., 2001). In this study, up-regulation of gene expression of aggrecan and type II collagen was consistent with increases in PG and collagen synthesis by extracellular adenosine. Since a previous study showed that mechanical loading could promote hydrolysis of extracellular ATP (Wang, et al., 2013), increase and accumulation of adenine derivatives such as adenosine is expected

in the IVD due to its avascular nature. Therefore, increases in PG and collagen synthesis and their corresponding up-regulation of gene expression found in this study suggest that mechanical loading may promote ECM biosynthesis of IVD cells via hydrolysis of extracellular ATP into adenosine.

With adenosine receptors expressed in most cells and organs, overwhelming evidence propose extracellular adenosine as a local modulator with protective action in the body (Fredholm, et al., 1994, Linden, 2005). Exogenously applied 2- chloroadenosine, an adenosine receptor agonist, in combination with endogenously released adenosine, have prevented damage to human vascular endothelial cells, suggesting that an increase in adenosine release could help to reduce inflammation at the site of injury (Cronstein, et al., 1986). In rats, a single daily dose of adenosine attenuated the joint injury associated with experimental arthritis (Green, et al.). In in- vitro studies, low doses of methotrexate, which is used to control inflammatory diseases such as rheumatoid arthritis, stimulated adenosine release from connective tissue and prevented neutrophil adherence to those cells. Therefore, the anti-inflammatory effects of methotrexate are suggested to be in part mediated by adenosine release promoted by methotrexate (Cronstein, et al., 1991). Furthermore, the involvement of A2A and A3 receptors has been reported to be imperative for the anti-inflammatory action of methotrexate (Montesinos, et al., 2003). Stimulation of the A1 adenosine receptor decreased inflammation and joint swelling and inhibited cartilage and bone destruction in a rat model of chronic arthritis (Boyle, et al., 2002). In murine models of arthritis, administration of low doses of A3 receptor agonists reduced inflammation with minimal damage to the bone, cartilage or joint in most of the treated

animals (Baharav, et al., 2005). Moreover, activation of adenosine receptors has been suggested to regulate maintenance of cartilage matrix (Tesch, et al., 2004). The aforementioned evidence indicates that A1, A2A and A3 adenosine receptors are involved in the maintenance of tissue integrity and in the anti-inflammatory actions of adenosine. Since inflammatory mediators are released from degenerated IVDs (Podichetty, 2007), extracellular adenosine may activate adenosine receptors to regulate inflammation and maintain homeostasis in the IVD. Inhibition of gene expression of aggrecan and type II collagen by the antagonists of A1, A2A and A3 receptors in NP cells and by the antagonist of A2A receptors in AF cells demonstrates that adenosine receptors are involved in the ECM biosynthesis of IVD cells.

In this study, intracellular ATP production in IVD cells was promoted by extracellular adenosine treatment. This finding is consistent with previous studies that reported that treatment with extracellular adenosine increased intracellular ATP levels (Chong, et al., 2009, Holland, et al., 1985). In human skin fibroblasts incubated with labeled adenosine, more than 90% of the label was found in the form of adenine nucleotides (mainly ATP) (Holland, et al., 1985). In Chinese hamster ovary cells, the majority of the adenosine added to the cell culture medium was reported to being taken up and metabolized into ATP (Chong, et al., 2009). In other studies, adenosine treatment has been reported to recover intracellular ATP levels after ATP depletion (Andreoli, et al., 1990, Wang, et al., 1998). In human umbilical vein endothelial cells, treatment with adenine nucleotides (including adenosine) promoted the recovery of intracellular ATP levels through adenosine uptake into cells after oxidant-induced ATP depletion (Andreoli, et al., 1990). In rats, adenosine

administration promoted ATP recovery in a dose-dependent manner during reperfusion after intestinal ischemia (Wang, et al., 1998). Furthermore, pre-incubation with adenosine demonstrated to preserve ATP levels in glial cells subjected to glucose deprivation and mitochondrial respiratory chain inhibition (energy-depleting conditions) (Jurkowitz, et al., 1998). Hence, those studies suggest that adenosine can mediate intracellular ATP production by adenosine uptake into cells and that adenosine is able to replete intracellular ATP levels of cells subjected to energy-depleting conditions. In the IVD, ATP production is fundamental since considerable amount of ATP is needed for protein synthesis especially PG biosynthesis that also uses ATP as a building block (Hirschberg, et al., 1998). Therefore, adenosine could be a potential backup metabolic pathway to produce ATP in the IVD when ATP concentrations required for biosynthetic activities are suboptimal.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 SUMMARY AND CONCLUDING REMARKS

Low back pain is a common physical condition that causes disability, morbidity, activity limitation, and economic loss especially among working people (Kelsey and White, 1976). Although its impact, the causes of low back pain remain to be elucidated. Correlation between progression of IVD degeneration and intensification of low back pain suggests that the IVD is a main aspect to consider. Therefore, investigating the processes associated with maintenance of the IVD may help on establishing new treatments for IVD degeneration and low back pain.

The mature IVD is the largest avascular tissue, and one of the most sparsely cellular tissues in the human body (Roughley, 2004). IVD cells are interspersed in a dense extracellular matrix mainly composed of proteoglycans, collagen and water. The interaction of the highly hydrated PG gel and the fibrillar collagen network determines the biomechanical properties of the IVD (Roughley, 2004). Therefore, maintenance of adequate rates of biosynthesis, breakdown and accumulation of ECM constituents by IVD cells determines the quality and integrity of the ECM and consequently, the IVD's functional properties.

Extracellular ATP triggers biological responses in a wide variety of cells and tissues and activates signaling cascades that affect cell membrane potential and excitability.

It has been demonstrated that compressive loading promotes ATP production and release by IVD cells, while a high level of extracellular ATP accumulates in the NP of the IVD. Since ATP is a powerful extracellular signaling molecule, extracellular ATP accumulation may regulate biological activities in the IVD. Moreover, it was also reported that compressive loading reduced extracellular ATP content in the center of healthy porcine IVDs , suggesting that compressive loading may promote ATP hydrolysis. Since adenosine can maintain ECM homeostasis and regulate different cellular activities via P1 purinergic receptors, hydrolysis of ATP into adenosine promoted by mechanical stimulation may influence cellular activities in the IVD via P1 purinergic receptors. Therefore, the main objectives of this dissertation were threefold: (1) to develop a non-invasive measuring system of ATP-induced membrane potential change of IVD cells; (2) to determine the relative gene expression change of aggrecan and type II collagen, the amount of proteoglycans and collagen deposition and intracellular ATP content in IVD cells treated with ATP; and (3) to determine the relative gene expression change of aggrecan and type II collagen, the amount of proteoglycans and collagen deposition and intracellular ATP content in IVD cells treated with adenosine. In order to achieve these aims, the following studies were carried out: (1) noninvasive measurements of transient membrane potential changes induced by exogenous ATP using the voltage sensitive dye di-8-ANEPPS (Chapter 3); (2) measurements of relative changes in gene expression and deposition of key ECM molecules and intracellular ATP production in IVD cells after ATP treatment (Chapter 4); and (3) measurements of relative changes in gene expression and deposition of key ECM molecules and intracellular ATP production in IVD cells after adenosine

treatment (Chapter 5). The most important findings from these investigations are discussed below.

6.1.1 Measurement of ATP-induced membrane potential changes in IVD cells

A noninvasive system was developed to measure ATP-induced changes in the membrane potential of porcine IVD cells using the potential sensitive dye di-8-ANEPPS and image processing techniques. The responses of NP and AF cells to ATP were examined in monolayer and 3-dimensional cultures. Stimulation with ATP showed distinct patterns and magnitudes of the membrane potential change response between AF and NP cells and between the two cell culture environments in which IVD cells exhibited different phenotypes. In addition, gene expression of $P2X_4$ purinergic receptor was found in both cell types. Inhibition of the ATP-induced response by PPADS, a non-competitive inhibitor of P2 receptors, suggests that ATP may modulate the biological activities of IVD cells via P2 purinergic receptors.

The ATP levels required to induce membrane potential changes was higher than the reported in electrophysiological recordings. There are two possible reasons for this difference. Firstly, the use of a voltage sensitive dye to measure membrane potential changes may not be as sensitive to precise measurements of the traditional patch clamp technique. Secondly, P2 receptors of IVD cells may be desensitized since high extracellular ATP level (~165 μM (Wang, et al., 2013)) was found in the IVD. Moreover, PPADS has been reported to have a modest inhibition effect at blocking $P2X_4$ (Soto, et al., 1996). However, in this study, PPADS showed to be very effective at blocking the $P2X_4$ receptor

in IVD cells compared to cells from different species. This could be explained by the variability of sensitivity to PPADS among different species.

6.1.2 The effects of ATP on the extracellular matrix biosynthesis of IVD cells

The effects of extracellular ATP on the ECM biosynthesis of porcine IVD cells isolated from NP and AF regions were investigated. The ATP treatment significantly promoted the ECM deposition and corresponding gene expression (aggrecan and type II collagen) by both cell types in 3-dimensional agarose culture. A significant increase in ECM accumulation was found in AF cells at a lower ATP treatment level (20 μM) compared to NP cells (100 μM), indicating that AF cells may be more sensitive to extracellular ATP than NP cells. NP cells also exhibited higher ECM accumulation and intracellular ATP than AF cells under Control and treatment conditions, suggesting that NP cells are intrinsically more metabolically active. Moreover, the ATP treatment also augmented the intracellular ATP level in NP and AF cells. These findings suggest that extracellular ATP not only promotes ECM biosynthesis via molecular pathway but also increases energy supply to fuel that process. Moreover, accumulation of extracellular ATP in the IVD regulated by mechanical loading suggests a mechanobiological pathway for regulating ECM production in the IVD.

6.1.3 The effects of adenosine on the extracellular matrix biosynthesis of IVD cells

The effects of extracellular adenosine on the ECM synthesis of porcine NP and AF cells were studied. Extracellular adenosine treatment significantly promoted intracellular ATP production and ECM deposition with up-regulation of aggrecan and type II collagen

gene expressions in IVD cells in 3-dimensional agarose culture. NP cells showed higher ECM accumulation and intracellular ATP than AF cells under Control and treatment conditions, suggesting that NP cells are intrinsically more metabolically active. Moreover, gene expression of P1 receptors was also found in both cell types, suggesting that adenosine receptors are involved in biological activities in the IVD. These findings suggest that hydrolysis of extracellular ATP promoted by mechanical loading may regulate ECM biosynthesis of IVD cells via adenosine formation.

6.2 RECOMMENDATIONS FOR FUTURE WORK

The overall objective of this research was to investigate the effects of direct stimulation of the purinergic pathway by extracellular ATP and adenosine on the ECM biosynthesis of IVD cells. Although the studies presented in this dissertation provide valuable insight into the mechanisms of ECM biosynthesis in IVD cells, there is still additional research left for fully understanding the ECM biosynthesis process in the IVD under the influence of extracellular ATP and adenosine. Therefore, recommendations for future work are described.

In Chapter 3, a non-invasive system for measuring transient transmembrane potentials changes was described. In this study, subcellular membrane potentials (e.g., mitochondrial membrane potential) were not taken into account, and it was not verified whether subcellular structures contribute to the overall effect seen in the measurements. However, the use of mitochondrial inhibitors in order to separate the mitochondrial and cellular membrane components of the fluorescence signal could be included in future studies. In addition, it has been reported that stimulus such as mechanical stress open hemichannels

(Baroja-Mazo, et al., 2013). Moreover, hemichannels allow permeability of molecules such as ATP (Harris, 2007). Different connexins such as connexins 26, 32, 37 and 43 are proposed to be involved in ATP release (De Vuyst, et al., 2006, Eltzschig, et al., 2006, Sonntag, et al., 2009, Zhao, et al., 2005) and also pannexins such as pannexin 1(D'Hondt, et al., 2011). Several purinergic receptors are involved in the opening of hemichannels, amplifying the purinergic signaling in a positive feedback loop manner (Bao, et al., 2004, Pelegrin and Surprenant, 2009). Therefore, the study of the interaction of pannexin/connexin hemichannels with purinergic receptors could be included in future studies with the use of hemichannels inhibitors. Additionally, it was found that concentrations of ATP higher than 100 μM were necessary to detect membrane potential change responses. Thus, it is recommended to improve the overall sensitivity of the system by using an image intensifier to amplify the input light-signal of the camera in order to detect small changes in the florescence intensity after ATP stimulation.

In Chapter 4, it was demonstrated that stimulation of IVD cells with extracellular ATP for 21 days promoted ECM biosynthesis by increasing PG and collagen contents while up-regulation of their corresponding genes was seen at the initial 16 hours of ATP treatment. However, temporal changes in biochemical content and gene expression profiles would be interesting to assess at multiple time points over 21 days. This would allow for a more thorough understanding of the process of ECM biosynthesis.

It was also found that the doses of ATP administered to IVD cells (i.e., 20 μM and 100 μM) promoted an anabolic response. However, more concentrations should be tested in order to identify a therapeutic concentration that maximizes the anabolic effects of ATP.

Furthermore, the doses of ATP and adenosine at which both nucleotides exert their maximal anabolic response on IVD cells should be investigated in order to compare ATP and adenosine effects on the accumulation of PG, collagen and intracellular ATP contents in IVD cells.

The effects of extracellular ATP on the accumulation of extracellular inorganic pyrophosphates, which are by-products of ATP degradation, need to be studied since purine receptors have demonstrated to regulate extracellular inorganic pyrophosphates in chondrocytes (Rosenthal, et al., 2010). Moreover, inorganic pyrophosphates are involved in the regulation of normal and pathological mineralization. Since the porcine model may not exactly simulate the conditions of human discs, future studies are required to confirm the findings of this study on human IVD cells. Furthermore, the IVD cells used in this study were from healthy juvenile IVDs. Therefore, future studies with cells from degenerated IVDs are necessary to examine if the findings of this study can be translated to cells from degenerated IVDs.

In summary, the recommended studies would enhance the findings of this dissertation, and would allow us to elucidate the biological effects of extracellular ATP and adenosine on the ECM biosynthesis in IVD cells, which can help to develop novel therapies for IVD degeneration and low back pain.

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