A Collagen Based Scaffold for the Repair of Annulus Fibrosus Defects

by

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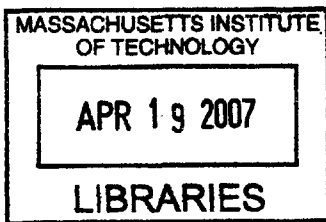
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ABSTRACT

The intervertebral disc (IVD) is the natural cartilaginous cushion found between the osseous vertebrae of the spinal column. It is an essential element for the flexibility of the spine but undergoes major degeneration with age, resulting in the loss of its functionality and in back and leg pain. Due to the large size of the IVD, its avascular nature and the difficulty for nutrients and waste particles to diffuse in or out of the IVD, damage and defects incurred by the IVD, be they natural or accidental, are very unlikely to heal and often trigger faster degeneration. The annulus fibrosus (AF) defines the outer boundaries of the disc and provides the structural integrity of the IVD. The purpose of this thesis was to evaluate the regenerative potential of a collagen type II scaffold on standardized defects of the AF. Specifically, this thesis evaluated *in vitro* the effects of the culture conditions, of a select group of growth factors, of the cross-linking method and of the biochemical composition of the scaffold, in the perspective of preparing a suitable autologous cell-seeded implant for a goat animal model.

Cross-linking method and the presence of GAG dramatically affected cell proliferation and new tissue synthesis, with the most promising results obtained with dehydrothermal treatment (DHT) cross-linking and with scaffolds containing 5 to 10% of chrondroitin-6-sulfate. Autologous cells seeded in this promising scaffold and cultured in a serum-free environment supplemented with a mix of Transforming Growth Factor-$\beta 1$ and Fibroblast Growth Factor-2, maintained annulus cell morphology, GAG and type II collagen synthesis, while exhibiting controlled contraction and a significant amount of new synthesized tissue just after a week in culture. Although culture in a rotating bioreactor showed encouraging results, lack of reliability in the replication of the experiments as well as uniformity in the resulting scaffold geometry and poor cell distribution in the scaffold, did not enable this culture condition to be a good candidate for implantation.

To investigate the regenerative potential of this implant, an autologous AF cell-seeded scaffold was cultured for 10 days in a DHT cross-linked scaffold in the serum-free culture medium mentioned above. At this time, the implant consisted in a loose matrix of connective tissue mostly composed of fibrochondrocytic cells and type I and II collagens. When surgically implanting this scaffold in a standardized defect of the annulus fibrosus at the L4-L5 lumbar disc level, results showed slight reduction in fibrous tissue and increase in the fibrocartilaginous tissues 2 months postsurgery compared to untreated

controls or controls where a scaffold without cells was used. However, there was no significant improvement in the gross morphology of the disc.

The results of this thesis recommend the use of an autologous AF cell-seeded type II collagen scaffold in the perspective of regeneration of defects of the annulus fibrosus. Future animal experiments should aim at locating the cells in the implant, evaluate the mechanical properties of the regenerated tissue and compare AF cells to stem cells that are easier to be harvested.

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This work is the result of five and a half years of my life. It would not be enough to only recognize the time and energy committed to this thesis, without acknowledging first how much I have learnt with and how grateful I am to the numerous and wonderful people who have helped me achieve this work.

After having written the pages that follow, I am now finishing my thesis with the acknowledgements, under the pressure of a deadline, so I wanted to apologize in advance for the people I will skip inadvertently due to sudden and unexplained memory holes.

During these few years of research, Professor Yannas and Professor Socrate, both members of my thesis committee, you have constantly brought your opinion, advice and critiques to this work, and helped me continuously make it better. For this invaluable input, I am very grateful.

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To the new and future generations of "labmates", who are the most likely to read this thesis completely, I wish good luck and welcome any of your questions. You will have the chance to work with a super boss... remember that!

MIT was also a source of wisdom and was rich in new friendships and I have to limit the coming list to the closest of them. To my ex-roommates first, among them, Sam, Wayne, Carlos and Julien, I wanted to express thanks for keeping our apartment in a tidy condition, for accepting to hold a soldering iron at 2am, for playing games with me, listening to me or for trying out my new dishes or desserts. To all the friends with whom I interacted so much during these five years, I just wish I would have stayed longer with you. I know that although our paths may seem to separate now, we will meet again in the

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CHAPTER 1 – GENERAL INTRODUCTION

1. Medical Problem

a. Low back pain

Low back pain is a painful condition that affects millions of people. It has been shown that 80% of the adults will experience back pain that will impair their activity. Indeed, back pain is the first cause of mobility restriction for people under 45 years old. This situation affects people's work and quality of life and leads to high health care and socio-economic costs ([1, 2]).

Although 90% of the people who experience back pain usually recover within 3 months, 1% of the US population is chronically disabled by back pain and 1% additional people are temporarily disabled by back pain. 10% of the people affected by back pain will become chronically disabled ([3, 4]).

b. Challenging diagnostics and understanding of pain

One of the biggest challenges when diagnosing the source of back pain is that often times, no specific problem can be found. In the rare cases where a lesion is identified, doctors often disagree on the treatment options.

Although low back pain can be associated with multiple other conditions or diseases, often times, the intervertebral disc (IVD) will show lesions making it the first tissue to be looked for back pain diagnosis. However, there is no direct obvious link between disc degeneration or lesions and pain symptoms: some degenerated discs can be completely asymptomatic whereas some painful discs may still appear relatively healthy ([5-7]). Besides, disc are often severely degenerated and even a highly trained eye will have difficulty differentiating between a young and an old disc ([8, 9]). Additional challenges arise from the natural variations across individuals, which lead to important issues to discriminate between age-related, premature degenerative or pathologic changes of the disc ([10]).

Despite these challenges, researchers still believe that disc degeneration is the strongest cause of back pain due to the loss of mechanical integrity that occurs during the degenerative process. Indeed, in several studies, disc degeneration and disc herniation are associated with back pain ([11]).

Pain was originally thought to occur due to the compression of the disc. This led several scientists to do thorough mechanical analyses of the spinal segments. When sciatica and back pain were found in a morphologically healthy and uncompressed disc, research started to focus on biochemical and cell-related changes ([12]). However, current diagnostic tools, including MRI or CT scans, only address the morphology of the disc and nearby bones, without revealing any information about the pain of the patient or the biochemistry or the disc.
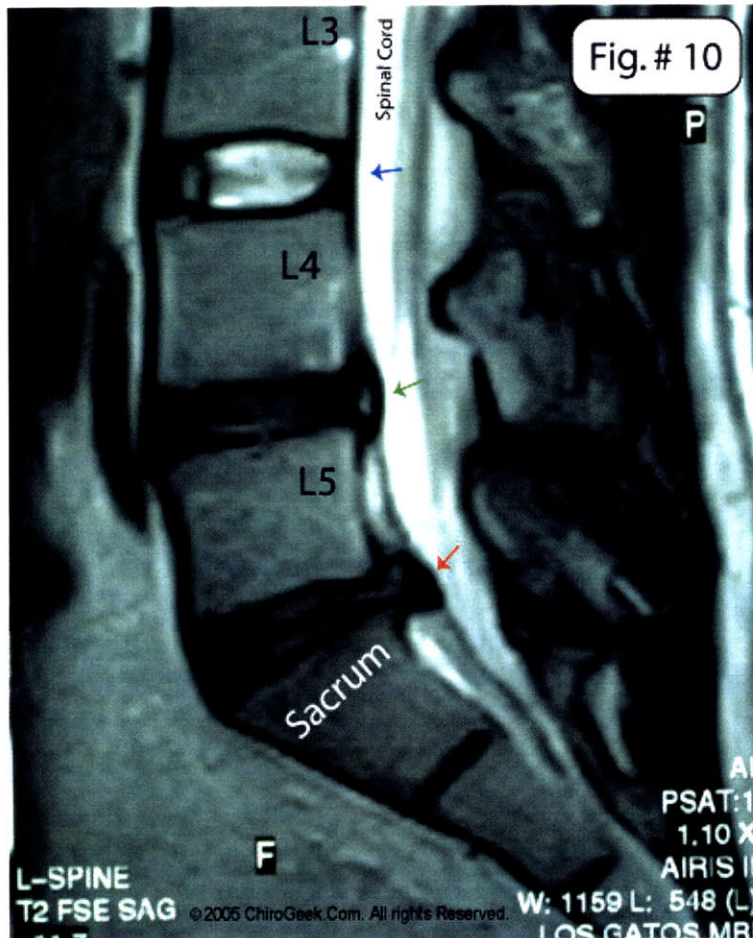
Fig. 1.1 MRI image of a sagittal view of the lower spine

c. Inefficacy of current therapy options

Back pain is treated at first by conservative means, mostly acupuncture, chiropractic treatment to correct spine misalignment, physical therapy to treat strains and to build back muscle strength.

Concurrently, drugs including anti inflammatory steroid drugs (aspirin, ibuprofen, naproxen, ketoprofen) or NSAID (COX-2 inhibitors), myorelaxants (cyclobenzaprine, methocarbamol), analgesic (Tylenol, anacin-3, etc.) are used to relieve the pain or relax the muscles. Drugs are either taken as tablets or injected by spinal injections.

If these options were not conclusive, surgery is another option: discectomy (removal of the nucleus), laminectomy (removal of the lamina), foraminotomy (expands openings through which the nerves go), spinal fusion (removal of the disc and implantation of a bone graft, cages, metal plates) in order to replace the disc by a bony structure to stabilize the spine and reduce the pain.

All these treatment options are aimed at treating the symptoms but often fail at this matter (see below). Besides, no surgical treatment addresses the problem by trying to regenerate what is often the source of the pain: the disc.

2. Tissue Description

a. Functional

i. Disc Morphology

The intervertebral disc morphology has been studied extensively and reported in Doers and Kang (1999), and in [13-15]. It is a very organized avascular ([16]) cartilaginous tissue with a complex matrix and relatively few cells. It comprises three specialized structures: the endplates, the annulus fibrosus and the nucleus pulposus.

- **Endplates**: occupying up to 90% of the inferior and superior interface between the disc and the adjacent vertebrae, the endplates have a similar structure to the one of the epiphyseal cartilaginous growth plates in bones. The endplates are essential in the reparative process because they facilitate the transport of blood, nutrients and waste molecules in and out of the disc.
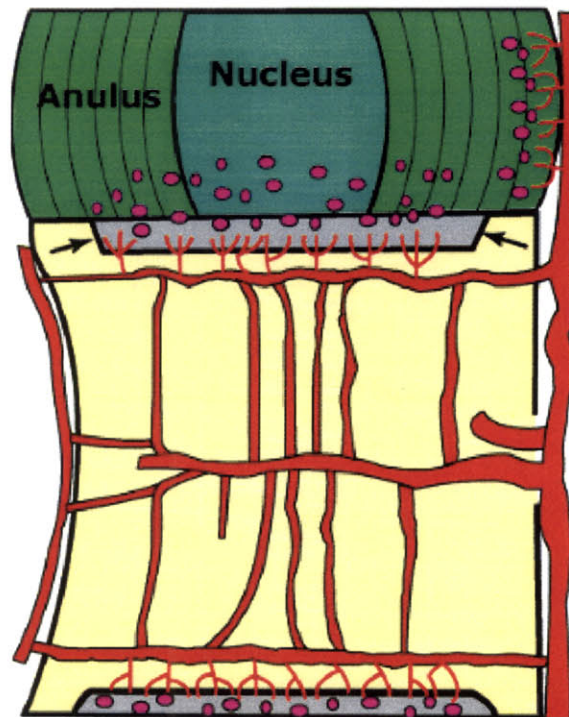
Fig. 1.2 Detail of the intervertebral disc and vertebral interface.

- **Annulus fibrosus**: consists of a strong network of parallel concentric rings of collagen fibers that are separated by elastin fibers ([17-19]). These rings, also called "lamellae", attach into the vertebral bodies inferiorly and superiorly through the end plates and connect to the posterior and anterior longitudinal ligaments.
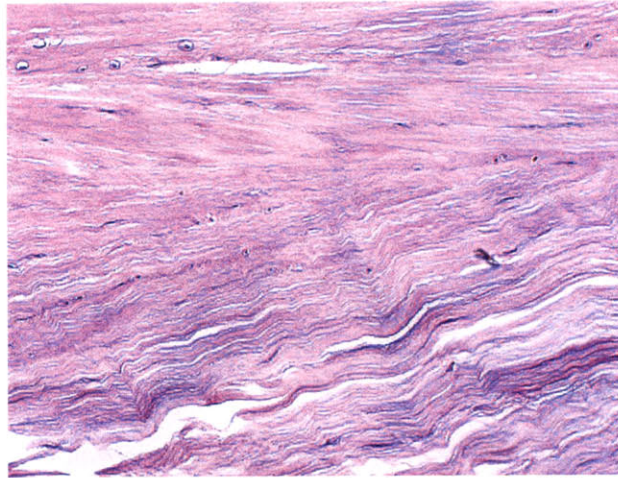
Fig. 1.3 Fibrocartilage of the intervertebral disc.
Collagen fibers are visible as well as a few annulus fibrosus cells.

- **Nucleus pulposus**: surrounded by the annulus fibrosus, the nucleus pulposus (NP) has a gelatinous structure and is composed of randomly organized fibers of collagen and of a highly hydrated aggrecan gel ([20]). It is the hydrated proteoglycans of the NP that give to the disc its load-bearing ability ([21]).
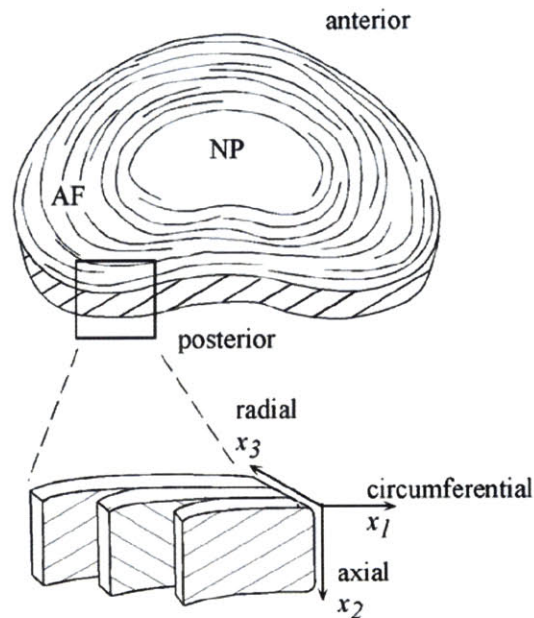
Fig. 1.4 Cross sectional view of the lamellar structure of the annulus fibrosus and of the orientation of the collagen fibers.
Source: http://www.uphs.upenn.edu/orl/research/bioengineering/afstructure.jpg

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ii. IVD functions and biomechanics

The strong organization of the disc enables movement and flexibility of the spine and supports the following movements: flexion, extension and rotation. The disc also absorbs the stresses from the body weight, muscles and ligaments.

These stresses follow a cyclical pattern and are very different during the day and at night ([22]). During the night where stresses are the lowest, nutrients and water flow back into the disc through the endplates and restore the original thickness of the disc. During the day, the disc is subject to high compressive stresses which result in pushing of the water out of the disc and decreasing of its thickness. Therefore, nutritive and hydration channels in the endplates are critical to maintain proper disc health, all the more important as the disc is an avascular tissue.

Loads on the disc are not only cyclical but they are also very complex, hard to measure and dependent on the age and strength of more than 20 other involved muscles, bones, ligaments and tendons. It is believed that these loads are "the most important contributing factor to the occurrence of low back pain and back injury" (Langrana, Edwards and Sharma). Large strains and displacements often occur after repeated loads and lead to permanent injuries of the disc and/or spine.

Mechanically, the hydrostatic pressure inside the nucleus is transferred radially to the annulus fibers which are then stretched or compressed. For example, when bending, the annulus in the anterior side is compressed whereas the posterior side is stretched, and conversely. To look at orders of magnitude, disc fibers strains are usually around 3% in compression and around 6% in extension. Axial rotation strains can go as high as 8%. The average day time load on an adult disc is of the order of 4000N (Humzah et al). Tensile strength of the annulus is around 150 to 500,000 Pa, however compressive loads of just 200,000 Pa could already cause the disc to rupture.

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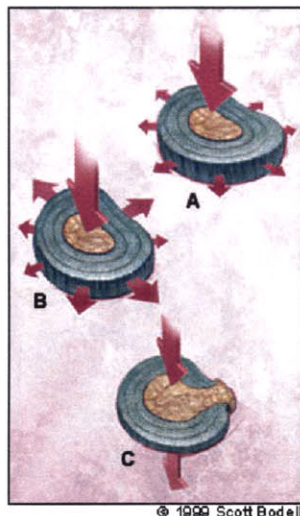
Fig. 1.5 Mechanical compression (A) and (B) of the disc and disc herniation (C)
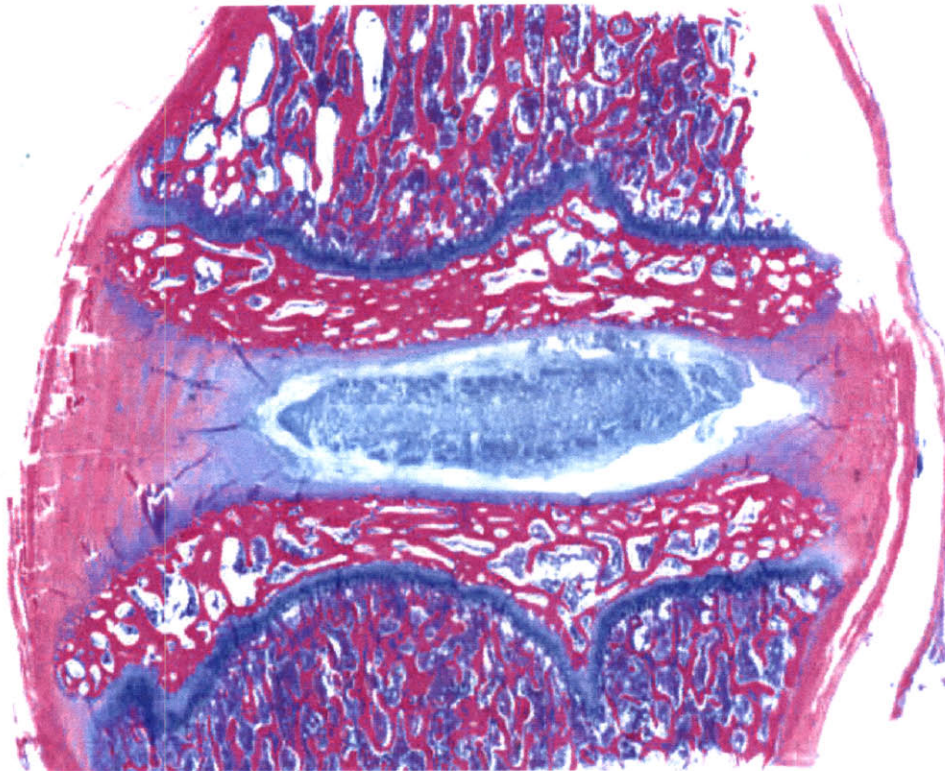
Fig. 1.6 H&E of a sagittal view of a normal disc. (source duke.edu)
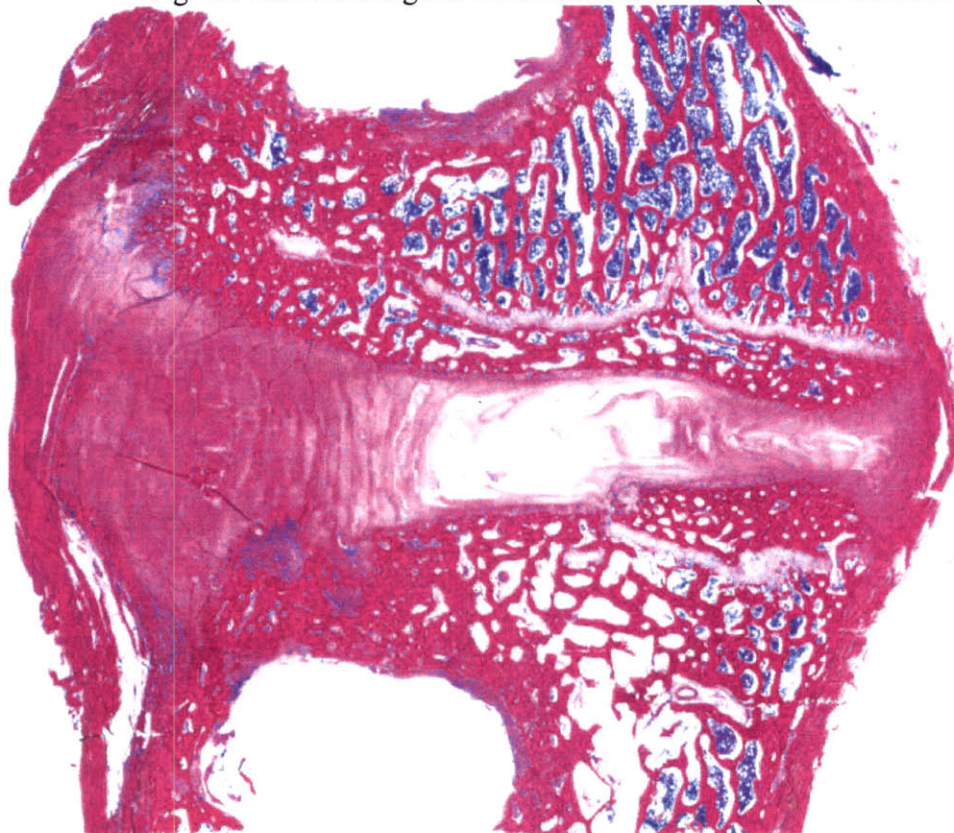
Fig. 1.7 H&E of a sagittal view of a degenerated disc. (source: duke.edu)

b. Extra Cellular Matrix Composition

The disc is a complex tissue with composition of each molecule that varies with its location in the disc. For example, elastin takes up only 2% of the dry weight of the annulus but it is found only between the lamellae of the annulus and nowhere else. Similarly, different types of glycosaminoglycans (GAG) or collagens have been localized at different locations in the disc. Collagen fibers orientation will also depend on the location in the disc.

i.   Collagens

The annulus, contains mostly type I and the nucleus mostly type II collagens ([15, 23-29]). These 2 collagens are important in the composition and load-bearing capacity of the disc. The conformation of the collagen fibers in lamellae and their orientation at an angle with the plane defined by the vertebral facets (see figure) participates in the structural properties of the disc. (Galante 1967). Several other collagen types have also been identified in the disc (I, II, III, V, VI, IX, X, XI).

ii.   Proteoglycans and water contents

Proteoglycans also play an important role in the load-bearing capacity of the disc. They are mainly found as aggregating molecules in the nucleus and due to their negative charges, they are able to retain water molecules. Water is an essential component of the disc with 88% of the nucleus made up of water at birth, 80% at 18 years of age and 65% at 77 (Naylor, 1970). This decrease in water content is linked to the decrease in the water-binding capacity of the GAG molecules and results in higher stresses in the annulus which are often the primary cause of tears. At the molecular level, it was observed that degenerating discs show an increase in decorin, biglycan and fibromodulin ([30]), which are smaller molecules, and a decrease in the chondroitin-4-sulfate, molecule which usually forms large proteoglycans ([31]).

iii.   Homeostasis

Regulation of the IVD tissue is done through a balance of anabolic and catabolic reactions ([32]) although it has been observed that catabolic reactions are prevalent and cause the degeneration of the disc. Several enzymes, cytokines, growth factors and enzyme inhibitors participate in changes of the metabolism of the disc cells. More will be explained below, but mainly, insulin like growth factor (IGF), transforming growth factor β (TGF-β), bone morphogenic proteins (BMP-2 and BMP-7) are all among these molecules acting as anabolic fashion ([33, 34]). Catabolic acting molecules include matrix metalloproteinase MMPs ([35-38]) such as MMP-3 and MMP-1, aggrecanases ([39]) such as ADAMTS-4 and other molecules such as the proinflammatory interleukin-6 (IL-6) or prostaglandin E2 (PE-2).

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iv. Cells characteristics

Cells comprise 1% of the total volume of the disc. In the annulus, most cells have a fibroblast like morphology and a more chondrocytic morphology in the inner annulus. Cells of the nucleus are usually chondrocyte like with cells that appear in lacunae ([14, 15, 40]). More about the cells metabolism will be described below.

3. Tissue Disorders

a. Morphological degeneration

Morphology of the disc is degraded early in life. As morphology and function of the disc are strongly linked to one another, disc function, both mechanical and physiological, is also degraded early. It is widely accepted that first degenerative changes start at two years of age and that most age-related changes will occur during the first decade of life. These changes are considered irreversible and currently without any effective treatment.

Age related degradation can be summed up this way: with age, the end plates become thinner, present crack and become calcified. As a result, water is lost in the nucleus which leads to changes in the proteoglycans structure and density of the nucleus ([41]). The disc then starts having clefts (fibrous tissue) and cracks, and tears form. Zones of vascularized granulation tissue with extensive innervations are then formed, extending from the outer annulus into the nucleus along the fissures ([42]). This shows that the repair response occurs from the extra discal area into the nucleus. However, as degeneration first occurs in the nucleus, the reparative response is too late to be effective. Loss of separation between annulus and nucleus is then observed in most people older than 20 years of age.
As these age-related changes are also seen in patients with degenerative disc disease, it makes it hard to differentiate effectively between age and disease factors.

These changes in the disc will in turn lead to a loss of functionality of the disc following the degradation loop: nucleus degrades => more stress on annulus => more strain on annulus fibers => tear in the annulus => nucleus bulges out => nucleus degrades even more => biomechanical performance of the whole segment is affected ([13]).

Although this loop pictures the degradation of the disc, there's no close link between pain and degeneration ([5, 43, 44]) and it is nearly impossible to differentiate pathologically degenerating disc to normally aging disc.

b. Specific extracellular degeneration

Most of the changes in the extracellular (ECM) matrix are attributed to the aging and pathology of degeneration ([45]). First, as the end plates calcify, nutrients intake and

waste disposals are more difficult. This leads to a lower pH in the tissue which is favorable to matrix degrading enzymes. As the tissue degrades, there is a reduction in aggrecan synthesis, resulting in a lower water-binding capacity of the nucleus ([46]) and hence in a reduction to absorb stresses ([47, 48]). Reduction and denaturation of type II collagen also occurs as well as increase in the synthesis of type I collagen in the tissue. Type IX and X collagens are also involved in the degeneration with a decrease in type IX and increase in type X ([49, 50]).

End plates calcify => Nutrients intake and waste disposal more difficult => lower pH in the tissue => matrix degrading enzymes activity is increased => reduction in aggrecan synthesis => lower water-binding capacity of the nucleus => reduction in the ability to absorb stress => increased stress and strain on annulus fibers => collagen fibers are torn

c. Cell transformations

Cell proliferation strongly decreases with necrosis and apoptosis ([51-57]) during degeneration. New cells are brought into the disc through the fissures forming granulation tissue. Disc cell metabolism is also strongly affected due to the loss of permeability of the end plates ([8, 58, 59])

4. Causes of Degeneration

The degeneration process is complex and not well understood process, limiting the efficacy of healing strategies. Different factors such as nutritional obstruction, injuries, mechanical stresses, cells senescence, changes in metabolism and other molecular mechanisms are thought to be the principal factors involved in the degenerative process. Newly discovered genetic factors are also starting to bring new knowledge on the process.

a. Injury / Mechanical

One of the main causes of future degeneration, mechanical injuries to the disc start by affecting the geometric organization and eventually lead to a loss of function and to further degeneration of the disc.

b. Biological

Degenerated discs are observed even in non injured tissues. Indeed, even early in life, IVD cells produce several matrix degrading enzymes that affect all the major constituents of the ECM, particularly collagens, proteoglycans and fibronectin.

The presence of **proteolytic enzymes** such as the matrix metalloproteinases (MMPs) was observed directly in the disc tissue ([60-65]) or in the mRNA levels

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expressing these molecules ([66, 67]). Examples of such molecules which affect directly the structure of the matrix [61] include collagenase (MMP-1) and stromelysin-1 (MMP-3). However, the actual effect of these MMPs is not completely understood and their presence could even prove beneficial in the regenerative process: indeed, if their presence in intact discs may be unfavorable, their presence in the prolapsed part of the disc could lead to its own digestion and to partial healing. What we know is that most studies have shown an increase of the levels of MMPs with the degree of degeneration of the disc, probably indicating a strong importance of these molecules in the degenerative process [65, 68-71]. However, whether these molecules were synthesized by the disc cells themselves or by inflammatory cells is still unclear ([72]) although one report showed an increase in the expression of several MMPs after the disc had been cut ([67]). Also, several studies report spontaneous synthesis of MMPs *in vitro* ([36, 63, 73, 74]), and that the levels of expression of these molecules could be stimulated by cytokines such as the proinflammatory cytokines IL-1 and necrosis factor-alpha ([75-78]) or by mechanical stresses ([79]). IL-1 and necrosis factor-alpha were also found in degenerated discs ([70, 80-84]).

The activity of the MMPs can be modulated to reduce the negative effects of these molecules by their inhibitors, also called TIMPs ([64, 70]).

There are also other collagens and proteoglycans degrading enzymes, such as cathepsins, which may be involved in age-related degeneration. Cathepsins were found in increasing amounts in degenerated disc and linked to end plates and annulus degenerations ([85-87]). The presence of cathepsins in the disc was also found to be upregulated by the presence of certain growth factors and proinflammatory cytokines ([88-94])

The levels of proteoglycans are also affected by certain molecules: Aggrecan for example is degenerated by aggrecanases, such as aggrecanase-1 also called ADAMTS4 ([70]), although once again, the effect of these molecules on the matrix is not fully understood ([39, 71]).

Growth factors have been shown to be involved in the degeneration of the IVD but also in the induction of pain [81, 95]. On the anabolic side, growth factors have been shown to increase mitogenesis, cell differentiation and extra cellular matrix synthesis. Among them, TGF-β was studied extensively because the molecule was detected in herniated disc samples ([96-98]) and because IVD cells also seem to be able to produce TGF-β on their own. The effect of TGF-β was to increase proliferation of the cells and proteoglycans synthesis and to decrease MMP-2 secretion ([99, 100]). Its strong expression as well as the presence of its receptors in the painful discs might indicate that TGF-β1 plays a key role in the repair of the injured annulus and in the subsequent disc degeneration ([42]). This also seems to be the case for the basic fibroblast growth factor, or bFGF or FGF-2. This growth factor is found next to the small blood vessels in herniated tissue leading researchers to think that angiogenesis could be one additional response to the disc to injuries ([97, 98, 101]).

Insulin-like Growth Factor-1 (IGF-1) is another growth factor produced by the disc cells ([33, 102, 103]). It was shown to have an anti-apoptotic function ([104]), to lower the levels of MMP-2 and to reduce tissue resorption ([34, 100]). Results also suggested that IGF-1 could be used by the cells to repair the prolapsed disc ([103]) and that the age-related decrease of IGF-1 could be the reason for the loss of proteoglycans in the disc ([33, 102]).

Finally, nerve growth factor (NGF), which was found only in painful discs and not in asymptomatic discs ([105-107]) could be a potential inductor for causing the pain,.

All these growth factors and cytokines influence the anabolic and catabolic processes of the disc and are thought to be important factors to explain IVD degeneration. Researchers think that this misbalance between the anabolic and the catabolic processes that lead to tissue degeneration. However, the exact biochemical processes are still not well understood.

c. Nutritional

Good health of the tissue depends on the good health of the cells, which depends strongly on nutrients supply and waste removal. Urban and Holm (The Intervertebral disc, Chapter 8, 1978) analyzed in detail the factors contributing to the nutrition of the IVD. As the disc is avascular, nutrients can only be supplied through the surrounding blood vessels. This was first shown by Brodin (1955) and confirmed by several other works. Most of the blood vessels are located in the end plates, in the nucleus region, where there exists a dense capillary bed, first visualized by Holm (1981). This capillary network is often considered to be compromised with age, limiting nutrition and waste removal to and from the IVD. This harsh environment leads to a more acidic environment more favorable to the activity of certain cathepsins, although MMPs activity is reduced at these low pH values (around 5).

d. Genetic

Recently, research has shown that heredity plays a dominant role in the degenerative process when certain genes are affected. This had been suggested by the study of real twins' disc degeneration ([108]). The first report of this was made when vitamin D receptor coding genes were associated with degeneration ([109]). Following were reports of problems associated with genes encoding collage type I, collagen type IX ([110-113]), aggrecan ([114]), matrix metalloprotease-3 ([115]), interleukin-1B ([116, 117]), interleukin-6, cartilage intermediate layer protein ([118]). Genetic factors were also studied in other studies ([109, 113, 114]).

5. Existing therapies

a. Therapeutic

Main therapeutic options aim at symptomatic relief of the pain. Drugs available on the market include:
- anti inflammatory (aspirin, ibuprofen, naproxen, ketoprofen, COX-2 inhibitors),
- myorelaxants (cyclobenzaprine, methocarbamol),
- analgesic (Tylenol, anacin-3, etc.).

Drugs are either taken as tablets or injected by spinal injections (for steroids or analgesic)

b. Physical therapy / Chiropractic

Physical therapy is used to treat back pain by building up patient strength, flexibility and endurance. Physical therapy can also be combined with chiropractic therapy used to correct spine misalignments, with heat/cold treatments, ultrasound, electric stimulation, massage or acupuncture.

c. Surgical

When conservative treatments have not successfully reduced patient pain, surgical options are considered. They include:
- discectomy (removal of the disc),
- laminectomy (removal of the lamina and loose tissues),
- foraminotomy (expands openings through which the nerves go),
- spinal fusion (removal of the disc and implantation of bone graft, cages, metal plates, etc. in order to replace the disc by a bony structure to stabilize the spine and reduce the pain)

These surgical options all have the drawback that none of them directly address the physiological repair and/or the restoration of the structural integrity of the disc.

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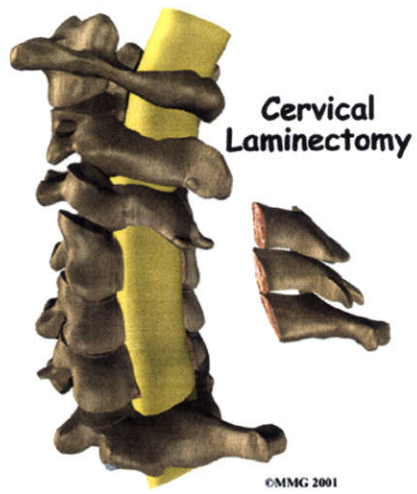
Fig. 1.8 Laminectomy
Source: eorthopod.com

Fig. 1.9 Discectomy
http://www.lowback-pain.com/CAPSTONEST06a.jpg
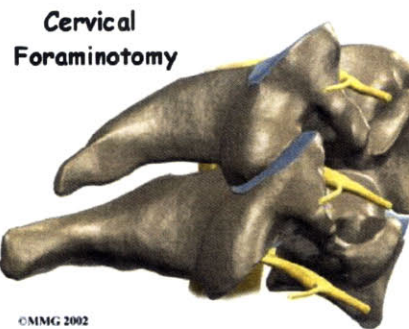
Fig. 1.10 Foraminotomy
Eorthopod.com

i. Fusion

When no other therapy has worked, surgeons can opt for fusion which replaces the disc by bone. Metal plates and rods are also used to stabilize the spine during the bone synthesis in place of the disc. While fusion is the last resort to eliminate pain and provide pain relief in the short term, spine fusion limits motion of the spinal segments and induces additional stresses on the adjacent segments. Problems of non unions were also reported in 5-35% of the cases by De Palma and Rothman (1968) as well as by Steinmann and Herkowitz (1992) and repeat surgeries used to avoid this bad outcome can fail in 40% of the time, as reported by Waddel (1979) and West (1991) and in [119]. 7-35% of the patients experience additional significant post surgery problems ([120-122]) due to altered biomechanics the adjacent unfused disc segments ([123, 124]). Fusion also seems to cause degeneration at adjacent levels ([125]).

Other problems also include facet joint syndrome, stenosis of the spinal root canal, spondylolisthesis (breakage of pars interarticularis and widening of the gap), and spondylolisis (breakage of the pars interarticularis).

Other surgical treatments, such as laminectomy, which consists in removal of any loose disc tissue and bony protrusions, also have unfavorable outcomes in around 11% of the cases ([126]). Discectomy, where the entire disc, loose or attached, is removed has severe complications due to the decrease in disc height. Complications include facet joint syndrome ([127]) and stenosis of the spinal root canal ([128]).

ii. Prosthetic

Over the past 10 years, following the success of arthroplastic devices for the hip and knee joints, disc prothetic devices have been developed to simulate disc function. The development of such devices was slowed by the complexity of the spinal segment, the nearby spinal cord and a poor understanding of the pathologic processes. The ideal

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prosthesis should restore disc height, reduce canal stenosis, restore spine mechanical properties. Products such as the Charite® or Prodisc® used as total disc replacements, have been studied extensively with nearly 10 years of follow-up [129-178]. There are also a few products which aim at nucleus replacement only. However, many of these products, although attractive on paper or FDA approved since 2004, fail in several outcomes: post operative complications directly related to the prosthetic device (3 to 6% of the operations) and residual leg and/or back pain are not uncommon. However, one should acknowledge that the Charite SB III ® device now presents strong advantages over fusion, and that long term failure rate has been very low.

Fig. 1.11 Charite® replacement disc
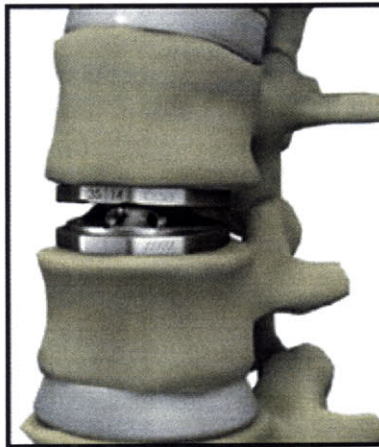
Fig. 1.12 Flexicore® replacement disc

d. Biological therapy

1. Review

Currently, all biological therapies target facilitation of interbody fusion but not the regeneration the biomechanical properties of the disc. With the recent discoveries on growth factors and other regulators of the disc, it becomes more attractive to use this knowledge to find suitable approaches to regenerate the functions of the disc. However, ambiguity in the knowledge and contradictory results make this task quite challenging. This task becomes even more challenging due to the unique properties of the disc: avascular, with a high complexity, low cell density and mitotic activity

Successful biological therapy could come from stimulating metabolism, proliferation and cell differentiation by introducing growth factors to enhance the often too poor existing anabolic process. Inhibition of certain growth factors, for example NGF, could reduce pain. Different growth factors, TGF-β1, OP-1, FGF-2, PDGF5, BMP-2 were injected directly ([179]) or have been synthesized by appropriately genetically modified cells ([180]). Although some positive results were noted, in a clinical perspective, it is unclear if the results obtained in animals could easily be replicated in the human IVD, particularly because human discs can be at a late stage of degeneration, with little cell density and with cells which may not respond to GF ([181]). Other challenges will include safety, side effects, duration of treatment, dosage and delivery methods ([182]).

Delivery methods will include: intradiscal injection, gene therapy approach, direct implantation of cells and finally, implantation of a biomaterial, seeded or not with cells. This last approach is the tissue engineering approach that we will consider throughout this thesis. Before developing this tissue engineering approach, let us review the other methods.

2. Intradiscal injection

This direct approach uses a simple needle to inject an active substance into the disc. Although very simple to execute, there are only a few reports of its use: in a rabbit lumbar IVD model, OP-1 was directly injected in the disc resulting in a restoration of disc height, stable for up to eight weeks after injection ([183-186]) and in a decrease in pain in a rat model ([187]). In a more recent study ([188]), a biochemical solution made from matrix components and biological components was injected in the disc with a follow-up of 13 months with positive findings. The matrix components were used to slow down the release of the bioactive components (growth factors mainly) and could also help regenerate the disc itself.

Direct injection methods are quick and simple but limited due to the low amount of cells, questionable cells healthiness and their response to growth factors. Even the approach that involves injection of a matrix is limited as to how much it could actually fill a defect. One also can worry about the time delivery of these substances and propose the use of slow release systems ([32, 182]. Thus, intradiscal injections are thought to be able to repair only slightly degenerated discs.

3. Gene therapy

Although this research did not involve gene modification of the cells, it is important to see that recent advancements in the knowledge of gene vectors have enabled the growth of gene therapy of the IVD cells. Usual gene therapy is done either by direct injection of the gene *in situ*, which is not very efficient ([189]), or by incorporation of the gene of interest in the cells and reimplantation of the modified cells ([180, 190, 191]). Techniques using viral and non viral transporters are used to transfer the genetic material into the cells. These techniques have greatly improved over the years and ([192, 193]) and could become more interesting in the treatment of IVD degeneration. Among the published studies ([66, 190, 191, 194-199]), successful reports have been made of transfected IVD cells with the gene for the human IL-1 receptor antagonist, TGF-β1, IGF-1, BMP-2 or TIMP-1. Although these studies show production of growth factors and anabolic or anti-catabolic effects *in vitro*, due to strong degeneration occurring in the IVD, it is questioned whether these results would replicate in the body, in a nutrient, glucose and oxygen deprived environment and at a lower pH. The fact that no animal model (see below) replicates the same nutrients transportation problem as the human disc, makes it even harder. Finally, ethical and political considerations could slow down future clinical trials dealing with genetically modified cells.

4. Direct implantation/injection of cells

Since 2002, four studies ([51, 200-202]) have been published on the direct injection of autologous cells to repair the intervertebral disc. The results seem to lead to the maintenance of disc height and of the hydration, compared to controls. This could have a potential benefit in clinical trials, although one can once again hardly extrapolate these results on situations where the annulus is already torn, as cells could be lost through the fissure.

6. Tissue Engineering Approach to IVD repair

a. Introduction to Main Challenges

Tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer and Vacanti).

Tissue engineering products still remain extremely scarce due to the complexity of the knowledge that their development requires. However, with the recent advancements in the knowledge of cell biology and biomaterials interactions with tissues, progresses in tissue engineering have been outstanding. Very recently, gene therapeutic techniques have been combined to yield interesting results in orthopedic research (see review by Jullig et al (2004) ([203])).

However, most tissue engineered products that are currently in phase I clinical trials do not have genetic therapeutics (cartilage, meniscus, ligaments, tendons, IVD). Tissue engineering approaches still present a lot of challenges for IVD regeneration:

- The IVD is an avascular tissue which has a very complex extra cellular matrix. Stresses that the disc need to carry, as well as poor nutrients transport make its regeneration even harder.
- Cells in the disc are often at an advanced senescence stage.
- Cells in the disc represent only 1% of the volume but are vital to the synthesis of new matrix molecules. Mechanical failure of the entire disc could depend on the cells biology. Replacing or regenerating decreasing cell population could correct matrix deficiencies and restore normal biomechanics ([106]). This stimulated the research on how to use, expand and reimplant cells from the patient's own disc, mostly because disc vascularity and reparative potential are low. Cells need to restore the appropriate matrix and its structural properties
- **Biomaterials** interaction with IVD cells are not well understood yet.
- **Suitable animal models** are hard to evaluate.

Below are presented more details on each of these challenges.

b. Type of cells to use

It would be natural to use disc cells for the regeneration of the disc. This is the approach of this research, although other types of cells, including chondrocytes, fibroblasts or mesenchymal stem cells could have potential.

Research has been done to transplant NP cells through a needle into the site of defect. The first experiment was done by [204] with injection of denucleated rat cells and showed that this technique could slow degeneration. Gruber in 2003 ([205]) introduced a technique for autologous disc cell transplantation demonstrating isolation, expansion and injection of annular cells.

However, using disc cells is strongly limited by the availability of such cells and their degree of degeneration. Besides, senescence of the cells is quite important and was studied in ([206]).

The use of stem cells could have several advantages such as cell availability and could solve senescence issues. Several reports aiming at increasing the cell population in the disc have been using stem cells ([205, 207-213]). Zhang *et al* injected MSCs into rabbit IVDs and found increase in proteoglycans and type II collagen mRNA compared to controls ([214]).

c. Source of cells: autologous / allogeneic / xenogeneic

The source of the cells is an important factor. The ideal choice would be to use autologous cells, i.e. cells coming from the patient himself. However, one is often confronted to the following problems:
- Number of cells: cells are harvested from a digested biopsy of the tissue. These cells are then expanded in vitro.
- Usefulness of the cells: cells need to be expanded in a suitable time frame, should keep their phenotype and should react potentially to growth factors if necessary

This autologous approach was used successfully the first time with autologous keratinocytes that were cultured in vitro and grafted onto victims of burns ([215]). Later appeared applications using autologous chondrocytes for articular cartilage repair ([216]).

Another possible source of cells is to use allogeneic cells, coming from donors other than the patient. This source would minimize problems related to shortage of cells in the patient and would enable the screening of suitable cells (in performance, variability and safety) that would be hard to obtain with autologous cells. A product using allogeneic cells is for example Apligraf, which uses keratinocytes to generate sufficiently enough cells to create a skin graft without any tissue harvesting on the patient ([217]). However, destruction of the tissue by the immune system will most likely occur if the cells induce an immune response. Fortunately, not all cells will be able to induce this immune response. For example fibroblasts and keratinocytes can be used as allogeneic sources without rejection. The fact that most engineered tissues are avascular also helps to limit immune response.

Finally, xenogeneic sources, coming from animals, have been used when autologous or allogeneic sources were not satisfactory for implantation. However, these sources require more treatment to minimize rejection by the immune system, which limits their use due to a higher cost. To minimize rejection, cell can for example be encapsulated in a semi permeable membrane [218].

d. Cell Harvesting

Cell harvesting is a procedure with a wide range of complexity. It can be as simple as the use of dermal punch to a complex surgery. In the case of the disc, cells of the intervertebral disc are not easy to access and can be either suctioned by needle aspiration or taken out through a small surgery. The biopsy that is taken out of the body is then digested and cells are isolated. When harvesting cells, one has to be concerned by:
- safety considerations for the patient
- localization of the source of the biopsy
- contamination from other population or viruses

e. Cell culture

Most importantly is that cells should retain their phenotype when cultured *in vitro*. This was showed by Gruber et al. Few studies have been done on disc cells, but results were conclusive on chondrocytes: once returned to the *in vivo* environment, they did integrate to the surrounding tissue ([13, 200, 219, 220]) successfully.
Other considerations include freezing of the cells, type of medium used to culture the cells, identification of the cell phenotype, consideration to mix different cell populations, etc.

f. Choice of scaffold

Scaffolds are useful for several reasons:
- scaffolds allow cells to grow in a 3D environment which will in turn synthesize a 3D tissue that will be easier to integrate *in situ*
- scaffolds allow cells to keep their phenotype contrary to monolayer culture for which often times, cells lose their phenotype (this is the case for example of articular chondrocytes)
- provide cell anchorage
- allows ECM molecules, cytokines or molecules to anchor onto the matrix
- provides an environment similar to the in situ ECM.

It has been shown that disc cells retain their phenotype up to passage 4 (Gruber) even in monolayer culture. The first stage, consisting of expanding the cells will therefore not influence the subsequent culture in the scaffold. Disc cells cultured in monolayer take a fibroblast like appearance ([221]). In a scaffold, they become rounded, form colonies, proliferate faster and synthesize more proteoglycans ([222]).
Diverse types of scaffolds have been developed including gels ([211, 223]), degradable polymer ([224]), hyaluronic acid ([225]), collagen, chitosan ([226]). Fibrin glue, agarose, chitosan gels were also used to entrap the cells.

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g. Seeding method

Seeding of the cells into the porous scaffold has always been a challenge and several techniques have been developed by tissue engineers. The goal is always the same: enable the cells to penetrate into the scaffold and attach to its pore walls. Often times, the first step is to optimize the geometric parameters of the scaffold (pore diameters, wall thickness, pore concentration, etc) to enable the cells to penetrate the scaffold. Problems commonly encountered in the literature are:
- No penetration: cells will not penetrate the scaffold or will remain attached to the outer part of the scaffold
- No attachment: cells will not attach to the scaffold
- Non uniform penetration: cells are non uniformly spread in the scaffold

h. Culture method

Culture of the cell-seeded scaffold in vitro remains challenging for diverse reasons. Although most cultures are still done in static plates, studies have shown the superiority of tissues cultured in environments like rotating bioreactors, rotating vessels, etc., which mimic the biomechanical environment of the cells or reduce shear stresses on the scaffold. However, little data has been obtained for cell-seeded scaffolds cultured in these bioreactor environments. Challenges pertaining to culture method are linked to:
- cell phenotype: will cells keep their phenotype once in the scaffold?
- amount/quality of tissue synthesized: will the tissue synthesized be superior in a static or in another environment?

Beyond static vs. dynamic cultures, a more important factor is the type of medium used and the supplements that it contains. In the perspective of preparing for FDA approval, one should keep in mind that only serum free media which contain well defined and controlled nutrients should be used.

i. Animal model

When selecting the best animal model for the evaluation of a tissue engineering product, particularly of the intervertebral disc, several factors play a critical role ([227-236]). They include:
- degenerative capabilities of the disc
- mechanical properties and mobility
- biochemical properties
- innervation
- presence of notochordal cells

Results have showed that there is currently no animal model that perfectly mimics the biomechanical structure of the human disc but that some models may be adequate for certain applications. In the case where we want to regenerate the disc, should we for example:

- Pick an animal model which has natural degeneration of the discs: although this would mimic the human IVD degeneration, the complete biochemical degenerative process could be very different. Besides, if regeneration does not occur, does this mean it would not occur in a human, and vice versa?
- Pick an animal model which does not present degenerating discs: would the knowledge of a positive repair be transferred successfully transferred to clinical trials?

j. Time of implantation

Certain studies report immediate implantation of the cell-seeded scaffold at the site of surgery whereas others first start by cultivating the scaffold *in vitro* for a certain amount of time before surgical implantation. This discrepancy can lead to dramatically different results as shown in this study ([237]) on a dog model for articular cartilage regeneration.

Time of implantation is challenging in several ways:
- Problems of implanting too early: this could lead to partial to no regeneration as the implanted tissue would have difficulty to be integrated in the existing tissue
- Problems implanting too late: the mature tissue would be less likely to be reshaped *in vivo* to form a living tissue, increasing the chance of rejection and the quality of synthesized tissue.

Finding the right time for implantation is difficult and there is currently no adequate technique for universally deciding when an engineering tissue is ready for implantation. Modeling such time would require a perfect understanding of the tissue synthesis, degradation and integration with the in situ tissue. This knowledge is unfortunately far from perfection.

k. Evaluation of success

Success can be evaluated by different parameters and can become a relatively subjective factor. Should "the reduction of pain" be the determining factor or should it be "the regeneration of the tissue"? These are important considerations that a researcher cannot ignore in the design of their experiments.

7. Objectives for this work

The overarching goal of this work is to prepare a tissue engineering implant and evaluate its regenerative potential in a standardized defect of the annulus fibrosus. The goal is not to synthesize an entire disc *in vitro* or *in vivo*, but to create a construct that will remodel *in vivo* to permit regeneration of a small defect.

a. Experiment 1: culture conditions (Chapter 2)

We have seen in the above review that culture conditions are important in determining the outcome in terms of the amount and type of tissue which is formed, and its homogeneity and the speed at which it can be engineered. With the promising results of chondrocytes cultured in rotating bioreactors vs. static cultures, and with virtually no other study aimed at evaluating the response of AF-cell seeded scaffolds, one could benefit from the study of the behavior of AF-cell seeded scaffold cultured in a rotating bioreactor.

Specific hypotheses for this work are:
- scaffolds cultured in bioreactors have more uniform distribution of cells and tissue synthesis than scaffold cultured in static plates
- scaffolds cultured in bioreactors will accelerate histogenesis.
- even in high (10%) serum supplemented medium, cells-seeded scaffolds cultured in the bioreactor will generate more new matrix than scaffolds cultures in static plates and low serum supplementation.

b. Experiment 2: effect of growth factors (Chapter 3)

As was seen in this introduction, growth factors are not only important as they may be necessary components for the healing response of the disc cells, they are also important in order to prepare a well defined medium (serum free) in the perspective of clinical trials. Prior studies have demonstrated the positive effects of selected growth factors on the proliferative and biosynthetic activity of AF cells in 2-D [34] and 3-D [238, 239] cultures. The purpose of this experiment was to evaluate the behavior of AF cells when grown in CG matrices cultured in medium supplemented with OP-1, TGF-β1, FGF-2 and with a combination of TGF-β1 and FGF-2.

Specific hypotheses for this work are:
- The above mentioned growth factors upregulate cell proliferation
- The above mentioned growth factors upregulate matrix biosynthesis rates.
- The above mentioned growth factors have a strong effect in generating a more uniform matrix

c. Experiment 3: scaffold composition and properties (Chapter 4)

Important design features of collagen scaffolds include collagen type (I vs. II), glycosaminoglycan (GAG) content, and degree and type of cross-linking. The objective of this experiment is to evaluate the effects of these three variables on the behavior of adult caprine and canine annulus fibrosus (AF) cells *in vitro*. In the present study the AF cell-seeded constructs were grown in serum-free medium containing TGF-β1 and FGF-2. These cytokines were employed based on work done in experiment 4 and demonstrating their beneficial effects on this cell type.

Specific hypotheses for this work are:
- the presence of GAG in the construct is necessary for the maintenance of cells and new matrix synthesis
- the way GAG was incorporated in the scaffold is important
- make up of the scaffold (type I vs. type II collagen) has a significant effect on the produced scaffold
- type of cross-linking (DHT vs. EDAC) determines new matrix production
- there is no significant difference between caprine and canine cultures

d. Experiment 4: animal model (Chapter 5)

In order to evaluate a tissue engineered product, it is necessary to find an adequate animal model and to follow up the implanted product for a certain amount of time. Although as seen before, there does not seem to exist a perfect animal model for the degeneration of the IVD, a goat model was chosen to evaluate the repair of a standardized defect in the L4-L5 lumbar annulus subject to one of the following treatments:
- no treatment
- implant of a scaffold alone
- implant of an autologous cell-seeded type II collagen-GAG scaffold cultured *in vitro* for 10 days before surgery.

Specific hypotheses for this work are:
- surgical implantation is feasible
- repair of the damaged disc is enhanced by the implantation of a cell-seeded collagen-GAG scaffold
- implant of a scaffold alone is not enough to regenerate the annulus fibrosus
- Scaffold will integrate into the surrounding tissue and produce the normal IVD ECM

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CHAPTER 2 – EFFECTS OF CULTURE CONDITIONS: CULTURE OF CELL-SEEDED SCAFFOLDS IN A ROTATING BIOREACTOR

1. Background on Culture Conditions

Culture conditions are extremely important when considering *in vitro* culture of tissue engineered implants before their surgical implantation. Indeed, even if the tissue engineered construct is bound to be remodeled in the body, *in vitro* remodeling has been shown to be critical for the success of a future implant and was demonstrated by Seliktar et al. 1998 on a blood vessel substitute. For that purpose, bioreactors, which define a chemically and mechanically controlled environment in which the implant can be cultured *in vitro*, were developed at MIT by Freed et al in 1993. These bioreactors have for main function to imitate nature and to improve the tissue engineering manufacturing process in the perspective of preparing a commercial product. To imitate nature, the bioreactor should try to bring the biochemical and mechanical signals that the tissue is expecting in order to synthesize a functional tissue *in vitro*. Freed and Vunjak Novakovic define a bioreactor as a "culture system that can perform at least one of the following four functions: (1) establish spatially uniform cell distributions on 3D scaffolds, (2) maintain desire concentration of gases and nutrients in the culture medium, (3) provide efficient mass transfer to the growing tissue, and (4) expose developing tissues to physical stimuli."

Several types of bioreactor systems have been used to evaluate their effect on cell behavior ([1-9]). They include the spinner flask, the rotating vessel (used in this study), the high aspect ratio rotating vessel, the rotating wall perfused vessel, the perfused column and the perfused chamber. Each of these different bioreactors were used with different types of cells and constructs. For a good review of these different systems, please see Principles of Tissue Engineering (Langer, Lanza and Vacanti), chapter 13.

IVD cells have been cultured in three-dimensional configurations, including alginate beads and gels [10, 11], agarose [12] or collagen-glycosaminoglycan (GAG) as implantable matrices [13-15]. Although scaffold make-up is a critical determinant of cell behavior, affecting the initial attachment of cells and other processes (see chapter 4), bioreactor culture systems have gotten much attention over the past ten years. Rotating-wall bioreactors, which randomize the gravity vector, were initially investigated as a model system in which to study microgravity [4, 5, 7, 9]. These rotating wall vessels allow for suspension of cultured substrates while reducing the shear stress applied on the constructs. They have demonstrated success in the cultures of different cell lines as well as in the culture of matrix constructs [1]. Several investigators [2, 3] have reported that bioreactor culture of chondrocytes with and without matrix scaffolds can also provide useful constructs for implantation into cartilage defects. This was also the case in a meniscus study ([16]). The work of Freed, *et al.* [1, 6-8], determined that the increased

mass transport rates of gases and nutrients provided by a rotating-wall vessel, could enhance the proliferation and biosynthesis rates of cell-matrix constructs by more than 50% as compared to static cultures, leading to the synthesis of constructs containing amounts of type II collagen and glycosaminoglycans comparable to normal articular cartilage. In terms of tissue generation, the bioreactor system has been shown to produce the greatest growth compared to other increased mass transport devices such as spinner flasks [8].

The promising findings of rotating wall bioreactor cultures for certain cell and tissue constructs prompted the current study to evaluate the behavior of AF cells seeded in type II collagen-GAG scaffolds in such a dynamic culture system. The cell-seeded scaffolds were cultured for 4 weeks in a rotating vessel in 10% fetal bovine serum (FBS). Comparison was made with seeded scaffolds in static cultures in well plates with 1 or 10% FBS.

Specific hypotheses for this work are:
- scaffolds cultured in bioreactors have more **uniform distribution of cells and tissue synthesis** than scaffold cultured in static plates
- scaffolds cultured in bioreactors **will accelerate histogenesis.**
- even in high (10%) serum supplemented medium, cells-seeded scaffolds cultured in the bioreactor will **generate more new matrix than scaffolds cultures in static plates** and low serum supplementation.

2. Materials and Methods

a. Experimental Design

Cells from 6 adult dogs were cultured and seeded separately on type II collagen-GAG scaffolds. Constructs were then allocated into three groups:
1) cell-seeded scaffolds cultured in static well plates in 1% FBS,
2) seeded scaffolds cultured in the rotating vessel in 10% FBS, and
4) unseeded controls placed in a static media with 10% FBS.

The constructs were cultured for 1, 2 and 4 weeks. For each time point, the DNA content, the accumulated GAG content, and the rates of total protein and GAG synthesis retained in the scaffolds were evaluated (n=6, each sample seeded with cells from one of the 6 dogs). Histology and immunohistochemistry for type I and II were performed at each time point (n=2).

Canine cells were used in this study because of the possible future implantation of the constructs in a canine model, and their successful use in prior work [13, 14].

The difference of FBS concentration (1% vs. 10%) in static cultures vs. rotating bioreactor cultures was decided in order to increase cell biosynthesis in static cultures and prevent cell detachment from the scaffolds in bioreactor cultures. The perspective of using serum free medium supplemented by growth factors also motivated this choice.

In a second experiment that mimics exactly the first one, static cultures were incubated in 10% FBS medium in order to bring a more direct comparison with the rotating bioreactor cultures.

Results of the two experiments are presented in this paper.

Free floating scaffolds in rotating bioreactor   Scaffolds in petri dishes
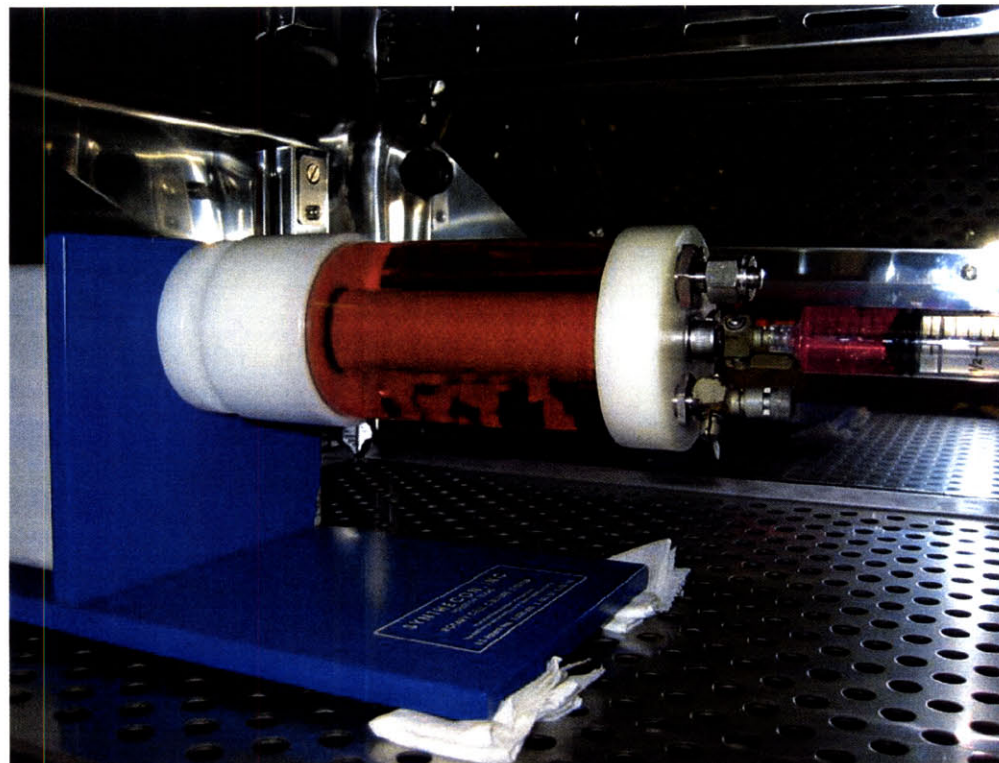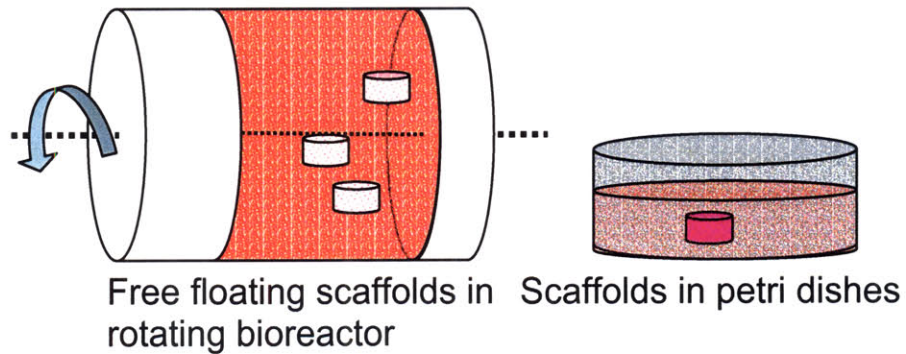
Fig 2.1 Bioreactor filled with medium. Floating constructs are visible.

b. Scaffold Manufacturing

Type II collagen-GAG matrices were produced by mixing porcine type II collagen (Geistlich Biomaterials, Wolhusen, Switzerland) and chondroitin 6-sulfate (C-4384, from shark cartilage, Sigma Chemical Co., St Louis, MO) at a concentration of 0.0105g per 20mL of collagen slurry. After 30 minutes of mixing, the slurry was poured in six well plates, 4mL per well and freeze-dried following a temperature ramping protocol from

room temperature to -40°C in about 3 hours [17]. Vacuum pressure was less than 200mTorr. Resulting pore diameter was evaluated to be 202±51μm, with a porosity of 91±2 and 6.7±2.2 % (w/w) of GAG content (mean ± standard deviation, SD). Freeze-dried sheets of the collagen matrices were approximately 3 mm thick.

The collagen sponge-like sheets were then cross-linked by dehydrothermal (DHT) treatment at 105°C for 24 hours. Nine mm diameter discs were punched from the sheets and the resulting samples were additionally cross-linked for 2 hours at room temperature by immersion in water containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; #E7750, Sigma Chemical Co.) at a concentration of 6mmol per gram of collagen and N-hydroxysuccinimide (NHS; #H-7377, Sigma Chemical Co.) at a 5:2 molar/molar EDAC:NHS ratio. Matrices were then washed twice and kept in distilled water overnight at 4°C.

c. Cell Processing

The AF cells were extracted from enzymatic digestion of the L1-L5 lumbar IVDs of 6 adult dogs (2-7 years old). Care was taken to exclude the nucleus pulposus and outer regions of the AF, which could clearly be distinguished in these canine IVDs. Enzymatic digestion was performed within 4-6 hours after harvest following a procedure detailed in [18], and cells from each of the 6 animals were expanded separately through 2 subcultures (*i.e.* to passage 2). Prior work [19] had demonstrated the phenotypic stability of 4th passage AF cells in two- and three-dimensional cultures, which justifies our use of passage 4 or lower AF cells for tissue engineering purposes.

Two million passage 2 AF cells were seeded onto the scaffolds, whose excess of water had been removed prior to seeding by blotting on sterile filter paper. One million cells in 25 μL of complete medium containing DMEM/F12 (#11320-033, Life Technologies, Grand Island, NY), 10% FBS (Hyclone Technologies, Logan, UT), 5 mL (1%) of antibiotic-antimycotic solution (No. 15240-096, Gibco-BRL), 10 mL (0.025g/L) of L-ascorbic acid phosphate and 1% of L-glutamine, were pipetted onto one side of the constructs. After 10 minutes, another one million cells were pipetted onto the same side. The cell-seeded scaffolds were then placed onto 6-well plates previously coated with a 4% (w/v) agarose gel and incubated at 37°C in 10% FBS.

d. Bioreactor culture

After 3 days in static culture, samples allocated to the bioreactor group were placed in the annular section of the bioreactor (model STLV, 250mL, SYNTHECON, Inc., Houston, Texas,). The bioreactor contained 250mL of 10% FBS complete medium described above. Static samples were placed in 12 well plates coated with agarose and incubated with either 1% (1st experiment) or 10% FBS (2nd experiment) as explained in the experimental design. This discrepancy between the first 3 days of culture was made in order to facilitate cell attachment and proliferation during the first 3 days of culture. This was particularly important for bioreactor samples, as prior experiment had shown that samples placed too early in the rotating vessel would lose most of their cells in the

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medium after a few days. The rotating speed of the vessel was chosen then regularly adjusted so as to maintain constructs freely suspended in the chamber [1]. Medium was changed every other day for constructs cultured in static petri dishes and half of the medium was replaced in the rotating bioreactor at the same time. Medium contained in the bioreactor was completely replaced when sacrificing the samples at 1, 2 and 4 weeks.

e. Radiolabel incorporation for the analysis of proteoglycan and proteins biosynthesis rates

The rates of synthesis of proteoglycans and total protein retained in the scaffolds over a period of 24 hours were assayed with the use of radiolabeled proline and radioactive sulfate ^{35}S (#NET 483, proline, L-[2,3,4,5-^{3}H]; #NET041H, sulfate ^{35}S; Perkin Elmer, Boston, MA). Cell-seeded samples were incubated in radiolabeled medium containing $10\mu Ci/mL$ of each isotope. After a period of 24 hours in incubation, unincorporated sulfate was removed by rinsing 5 times 15 minutes at 4°C with a solution of phosphate buffered saline supplemented with 0.8 mM Na_2SO_4 (anhydrous sodium sulfate, Sigma Chemical Co.) and 1.0 mM L-proline (P8449, Sigma Chemical Co.). The matrix discs were then frozen at -20°C, freeze-dried thoroughly and digested at 60°C in 0.125 mg of papain per 6.25 mg of dry sample. 100mL of the stock papain (#P3125, Sigma Chemical Co.) buffer solution was composed of 2.5 mL of 0.5M dibasic stock solution, 18 mL of 0.5M monobasic stock solution, 80 mL distilled water, 88 mg L(+) cysteine HCl, and 186 mg disodium ethylenediamenetetraacetate (EDTA, Fischer S-657), with the pH adjusted to 6.2 with a few drops of concentrated hydrochloric acid.

Radiolabel digests were assayed for radioactivity by combining $100\mu L$ of digest with 2mL of scintillation fluid (Scintiverse II, cat #SX12-4, Fisher Scientific, Fair Lawn NJ), and counted for 3 minutes in a liquid scintillation counter. Values of retained GAG and total protein synthesis rates were extrapolated from counts per minute using known amounts of radiolabeled medium. Mean counts per minute of unseeded control matrices was always negligible, showing the efficacy of the washing to remove all unincorporated isotopes. Therefore no adjustment of the results was required to account for the controls.

f. DNA and GAG accumulation

To normalize the results, dry weight was measured after freeze-drying of the constructs. DNA quantification was performed using the Hoechst dye (#33258, Polyscience Inc., Northampton, UK) assay [20] from the papain digested samples, the amount of DNA being extrapolated from the standard curve of calf thymus DNA. Average amount of DNA content of unseeded scaffold was subtracted to each sample to obtain the net DNA content of each scaffold. Number of cells could be estimated using an amount of DNA of 3.6pg per canine AF cell, which had been measured by determining the DNA content of aliquots of known numbers of cells digested in papain following the same protocol.

The total GAG content of the cell-seed constructs was determined using a dimethylene blue (DMMB) method [21]: A 100 μL aliquot of the papain digest was

mixed with 2 ml of the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The total amount of GAG was extrapolated from a standard curve of shark chondroitin sulfate. GAG content of unseeded constructs was also evaluated and subtracted from the total GAG content of the cell-seeded constructs to obtain the mass of newly synthesized accumulated GAG. This figure was then normalized by the dry mass of each construct to obtain the % mass of newly synthesized GAG. Previous studies have shown that it is often hard to quantify the amount of newly synthesized collagen in the scaffold as the make up of the scaffold is primarily collagen itself.

Due to the adjustment with the unseeded scaffolds, negative values of accumulated GAG, % mass of newly synthesized GAG or accumulated GAG per unit mass of DNA can indicate that the unseeded scaffold contained more GAG than the cell-seeded construct. This could be due to either a faster degradation rate of the cell-seeded scaffold, or a degradation or release of the initial GAG contained in the scaffold in cell-seeded constructs.

g. Histological Evaluation

Selected samples of cell-seeded scaffolds were fixed in 10% buffered formalin for 3 days. Specimens were then processed in a tissue processor (Hypercenter XP, Shandon) and embedded in paraffin. Sections, 7 μm in thickness, were cut along the horizontal plane of the scaffolds. The microtomed sections were stained with hematoxylin and eosin, safranin-O (for proteoglycan), and Masson trichrome (for collagen). Slides were cover-slipped and sealed with cytoseal 60 cell mounting medium. The following features were examined by normal and polarized light microscopy: cell morphology, cell distribution, cell layers on the surface of the disc, collagen fiber organization, and GAG distribution.

Immunohistochemistry for type I, II and III collagen was performed on selected sections; previous studies had shown that the collagen type II of the matrices themselves did not stain positive for type II if the matrices had been cross-linked with the EDAC beforehand. Deparaffinized and rehydrated sections were digested for 1 hour in 0.1% protease XIV, followed by blocking of non-specific binding with 5% horse serum. The primary antibody (CIIC1, mouse anti-chick type II collagen monoclonal antibody or C1D8, mouse anti-chick type I collagen monoclonal antibody, Developmental Studies Hybridoma Bank, Iowa City, IA) was diluted 1:20 and applied for 1 hour. Negative controls were incubated with a universal mouse negative IgG (N1698, Dakocytomation, Carpinteria, CA) diluted to the same protein concentration, instead of the primary antibody. A biotinylated secondary antibody (horse anti-mouse IgG, Sigma) was applied for 45 minutes, followed by quenching of endogenous peroxidase with 3% hydrogen peroxide. Labeling was detected with an avidin-biotin complex (ABC kit, Vectastain, Vector), and diamenobenzadine (DAB, Vector). Counterstaining was performed using Harris hematoxylin for 2 minutes followed by 5 minutes wash.

h. Statistical Methods

The sample size of 6 was based on a power calculation that sought to distinguish a 30% difference in the means of two groups for certain measurements (*e.g.*, 100% versus

70%), each with a 20% coefficient of variation (for $\alpha=0.05$ and $\beta=0.80$); Single- and multiple-factor analysis of variance (ANOVA) or Student t-tests were used to assess the effects of the duration of the culture and of type of culture on all of the measured variables. The Fisher least squares protected difference (LSPD) post-hoc test was also utilized for selected analyses using StatView (SAS Institute Inc., Cary, N.C.). Data are presented with the error bars representing the standard error of the mean.

3. Results

a. Macroscopic Examination

In both experiments (scaffolds cultured in 1% or 10% FBS), most cell-seeded scaffolds cultured in static cultures remained fragile and sponge-like during the time of the experiment, slightly less transparent than the non-cell-seeded matrices. However, a few of them changed their appearance becoming white, shiny and resistant to forceps pressure. There did not seem to be a visible difference between the static scaffolds cultured in 1% and in 10% FBS. In prior studies where scaffolds had been cultured under the same conditions, a greater percentage of scaffolds had shown that cartilaginous appearance after 4 weeks of culture, showing the variability of these experiments, the provenance of the cell source, the passage number and FBS batch.

In contrast to the static culture observations, scaffolds cultured in the rotating bioreactor rapidly changed their appearance and started to become cartilaginous after 2 weeks of culture. After 4 weeks, scaffolds began to aggregate, making it difficult to separate them, even with a scalpel blade (Fig. 2.2). This resulted in non uniform shape as seen on the micrographs. In the repeated second experiment, scaffolds cultured in the bioreactor did not appear as cartilaginous and did not aggregate. However in both experiments, scaffolds slightly bent under the flow of medium and took a dome shape after 4 weeks of culture.
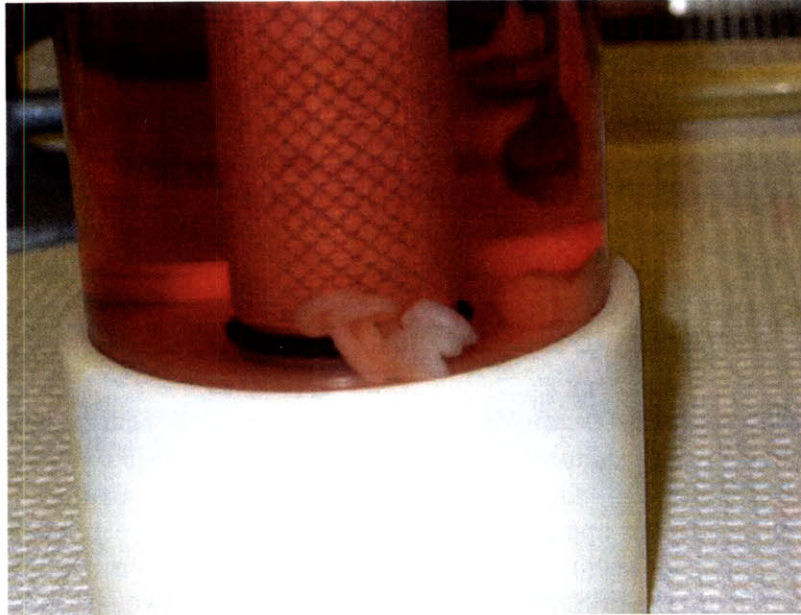
Fig 2.2. Bioreactor and constructs lumped together after 4 weeks.
Note the cartilaginous aspect of the constructs.

b. Cell Proliferation and Mass of the Constructs

In the first experiment, the quantity of DNA after one week was similar in both the bioreactor and 1% static cultures. If total DNA slightly decreased over the 4 weeks of culture in the 1% static culture, there was a significant 2-fold increase ($p<0.0001$) in the DNA of the bioreactor constructs between 1 and 2 weeks. Using an estimated DNA content of 3.5pg per cell, as was evaluated in previous work, this translated into an increase from 500,000 to 1 million of the number of cells in bioreactor constructs (Fig 2.3). Dry masses of the scaffolds followed the exact same trend: static cultures had constant masses over time whereas bioreactor cultured construct masses increased significantly to a value approximately 60% higher after 4 weeks. It was interesting to note that the % of mass DNA increased from 1 to 2 weeks from 4 to 8% and that this % decreased to 5% after 4 weeks showing that cells did not account as much in the scaffold make up as during initial periods.

In the second experiment, initial cell attachment and proliferation over the first week of the culture was much greater, although the cell line, passage number and animals were exactly the same. This resulted in an initial DNA content of about 2 million cells per construct after 1 week in either the static at 10% FBS or the bioreactor cultures, a much higher number than during the first experiment. Once again, number of cells decreased over the 4 weeks of culture in the static cultures, reaching a terminal value of just less than a million cells. The bioreactor constructs experienced a slight increase in number of cells between 1 and 2 weeks and a dramatic ($p<0.0001$) decrease after 4 weeks with just 500,000 cells left in the scaffolds. In that experiment, the % of mass of the scaffold that represents DNA followed the same pattern as the mass of DNA: static cultured experiments decreased from 12% at 1 week to 8 and 6% at 2 and 4 weeks. Bioreactor constructs DNA content decreased from 16% to 5% after 4 weeks.

60
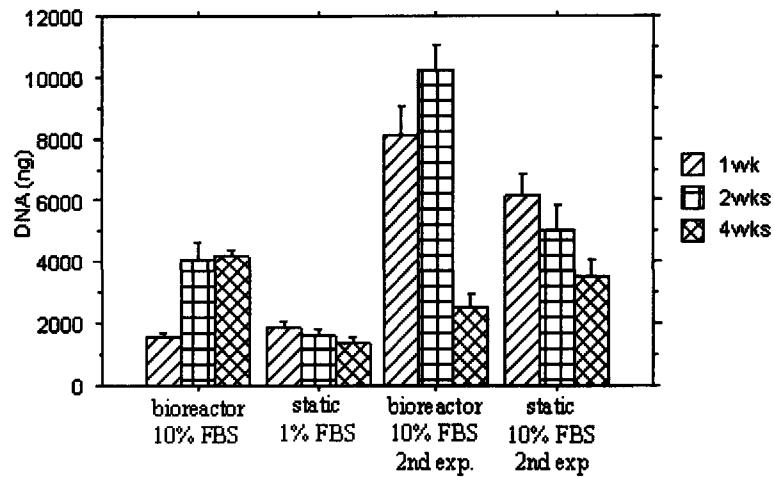
Fig 2.3 Mass of DNA
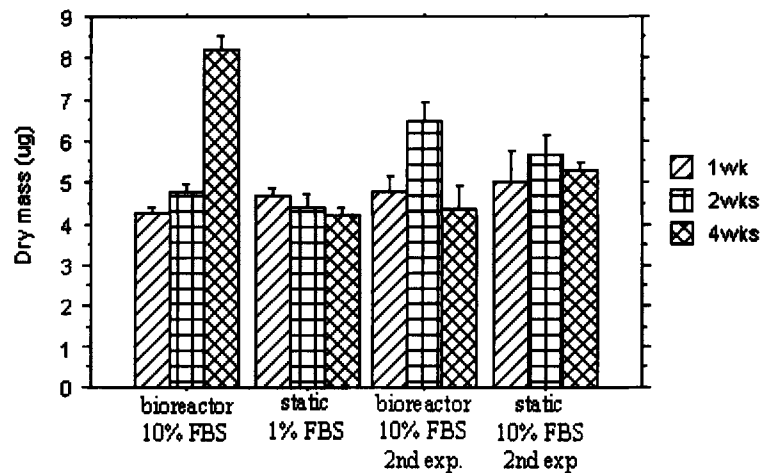
Fig 2.4 Dry mass of the constructs in the 4 different groups of scaffolds

c. Rates of Biosynthetic Activity

In both experiments, time and culture conditions (static vs. bioreactor) were significant factors affecting GAG and protein biosynthesis rates, as revealed by two-factor ANOVA ($p<0.001$; Fig. 2.5 and 2.6). In bioreactor constructs of the first experiment, proteoglycan synthesis rates reflected in retained GAG normalized to DNA content continuously increased during the culture ($p<0.0001$, power=1), reaching a value at 4 weeks more than twice higher than the static culture (Fig 2.6). The latter had a 2-fold increase from 1 to 4 weeks in these 1% FBS cultured samples. In the second experiment, there was no noticeable change neither in the 10% FBS constructs nor in the bioreactor constructs. The absolute values of GAG synthesis rates were significantly higher in the

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bioreactor samples of the first experiment, whereas these values were not significantly across the static cultures in the first and second experiment.

In the first experiment, after 1 week, the bioreactor group had a slightly higher protein synthesis rate than the static groups (Fig. 2.5). Biosynthesis decreased continuously between 1 and 4 weeks in both groups reaching a terminal value around 5 times lower than the value at 1 week. At 4 weeks, there was no significant difference between the two groups, showing no noticeable effects of the culture in bioreactor in increasing protein biosynthesis relative to static group. Two-factor ANOVA showed that time and culture conditions had significant effects on the biosynthesis rate ($p < 0.0004$). In the second experiment, absolute values of protein biosynthesis were significantly lower than in the first experiment and static cultures showed higher rates than bioreactor cultures.

These discrepancies across the two experiments show the variability in the repeatability of experiments involving a rotating bioreactor.
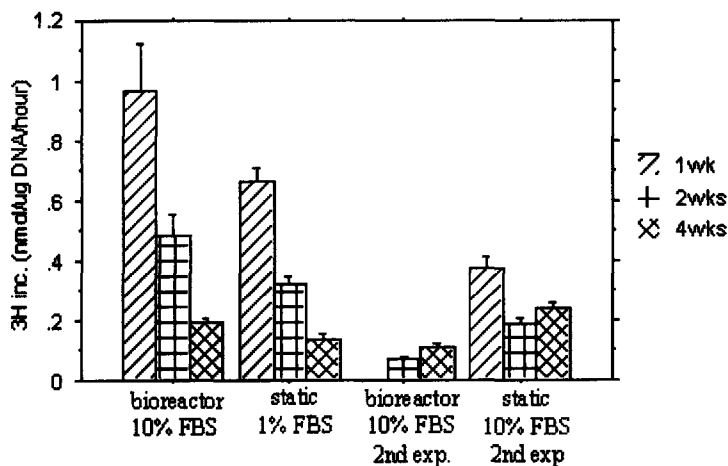
Fig 2.5 Rates of synthesis of protein. Normalized rates of nmol 35S incorporated per µg of DNA, per hour
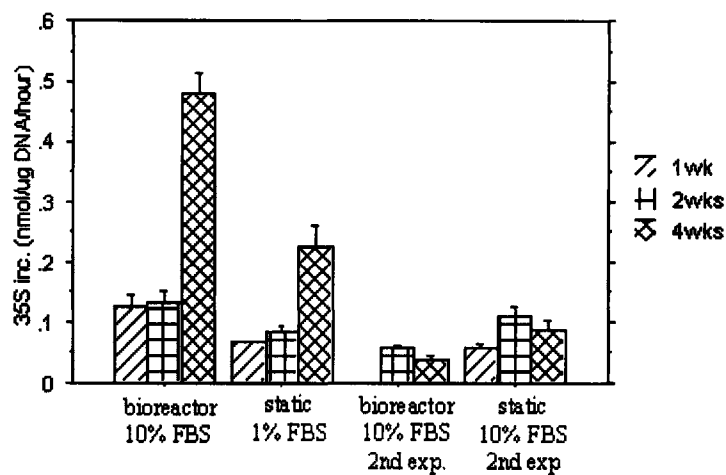
Fig 2.6 Rates of synthesis of GAG. Normalized rates of nmol 35S incorporated per μg of DNA, per hour

d. Accumulated GAG

During the first 2 weeks there were no notable differences among the groups with respect to the amount of accumulated GAG (Fig. 2.7a). Bioreactor samples of the first experiment, after 1 week degraded faster than the rate of accumulation of GAG, explaining the negative accumulated GAG value. After 4 weeks, there was a dramatic increase in the amount of accumulated GAG recorded for the bioreactor group representing a 3- to 4-fold increase over the static groups of the two experiments and the 2-week bioreactor samples (Fig. 2.7a). However, this increase was not to be noted in the second experiment.

When normalizing the accumulated GAG to the DNA content, bioreactor samples accumulated about 30% more GAG per cell than the 1% FBS constructs at 4 weeks (first experiment) (Fig. 2.7b). After 4 weeks, the constructs of the second experiment had the lowest GAG accumulation per cell, with values more than twice lower than in the 1% FBS controls constructs of the first experiment. When evaluating the amount of mass of the construct that is GAG, after 4 weeks, GAG in the bioreactor samples accounted for 1.5% (first experiment at 4 weeks) or 1% (second experiment at 2 weeks) of the total mass of the scaffolds, whereas this value was between 0.5 and 0.7% in the static cultures (Fig. 2.7c).
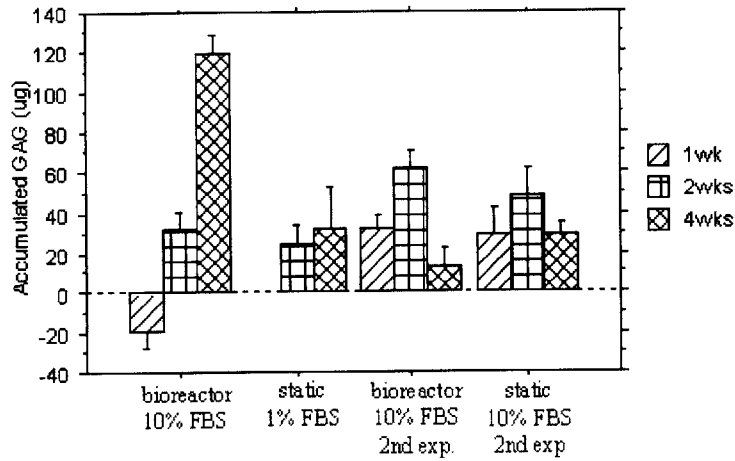
Fig 2.7a Accumulated GAG
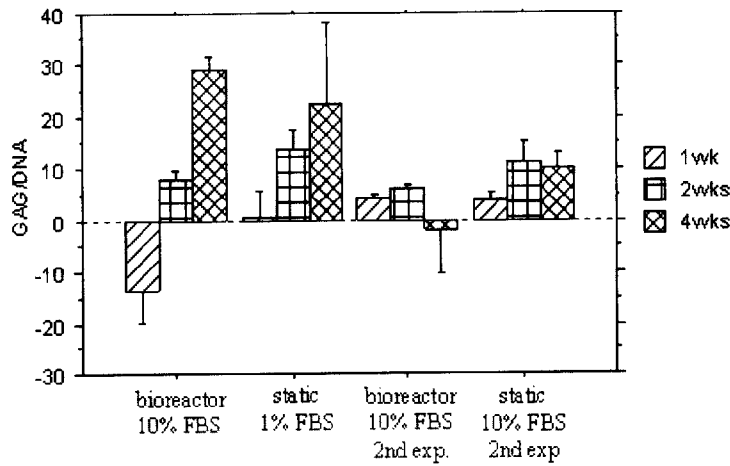
Fig 2.7b Accumulated GAG (ug) / DNA mass (µg)
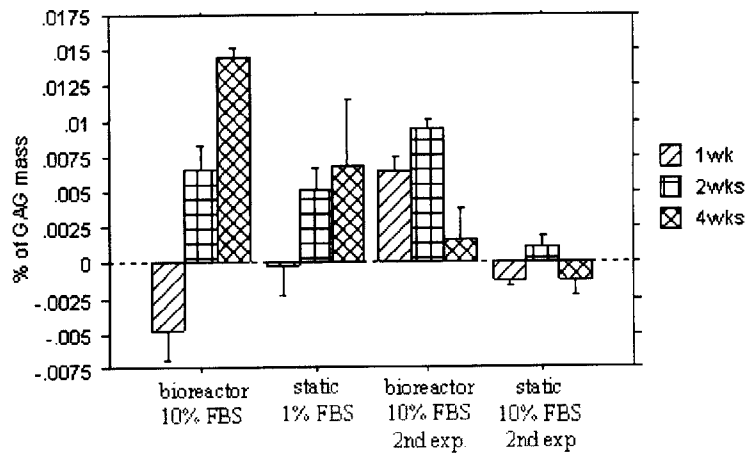
Fig 2.7c Percentage mass of newly synthesized GAG / total Mass % of GAG over total dry mass

e. Histology and Immunohistochemistry

Histology revealed that cells were distributed throughout the collagen-GAG matrices, although in bioreactor constructs, cell density was usually higher on the periphery of the scaffolds than in their centers (Fig. 2.8 and Fig 2.11). With increasing time in culture there was an increase in the amount of newly synthesized extracellular matrix in the pores and a reduction in pore size. This newly synthesized matrix appeared as thin filamentous material. Cells could be found suspended from these filamentous cables as well as attached to the walls of the scaffolds. Cells appeared in elongated and stellate morphologies. After 4 weeks in culture, samples cultured in static culture presented less apparent matrix than bioreactor samples.

As the time in culture increased, type I immunohistochemistry revealed the increasing presence of newly synthesized collagen type I in the matrices cultured in the bioreactor (Fig 2.8 and 2.9). This increase was not noticed in samples in static culture, for which type I immunohistochemistry stained negative. In bioreactor samples, birefringence of the collagen fibers could be observed demonstrating the high degree of organization of the collagen fibrils (Fig 2.12).

Safranin-O staining revealed that GAG was principally located nearly all over the scaffold for bioreactor samples. However, no significant amount of GAG was observed within the scaffold. For static constructs for which the newly synthesized matrix was not very dense, most GAG was located in the region surrounding the cells.

Of note was the formation of tissue-like structure in the bioreactor scaffolds after 4 weeks of culture (Fig 2.10a and 2.10b). In some cases this tissue structure appeared to be displacing the collagen-GAG scaffold.

300um
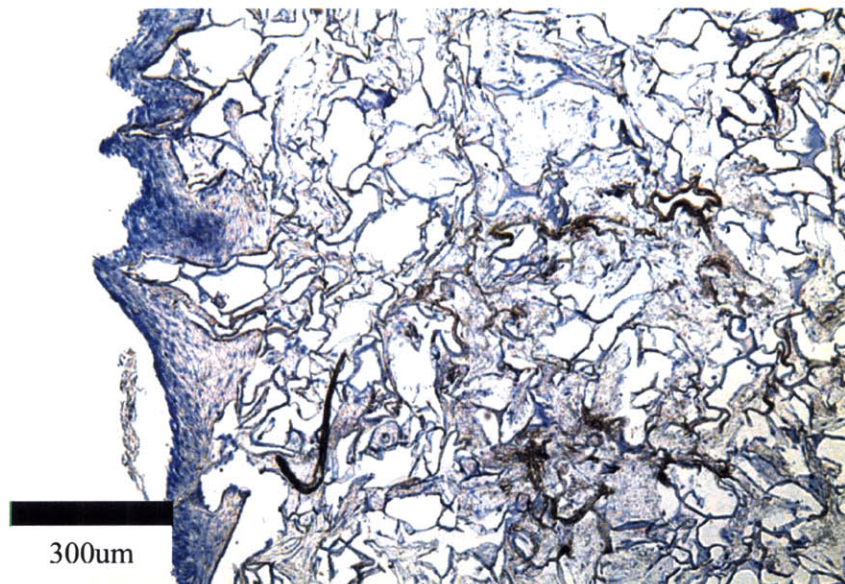
Fig 2.8 Bioreactor, 4 weeks, type I collagen immunohistochemistry.
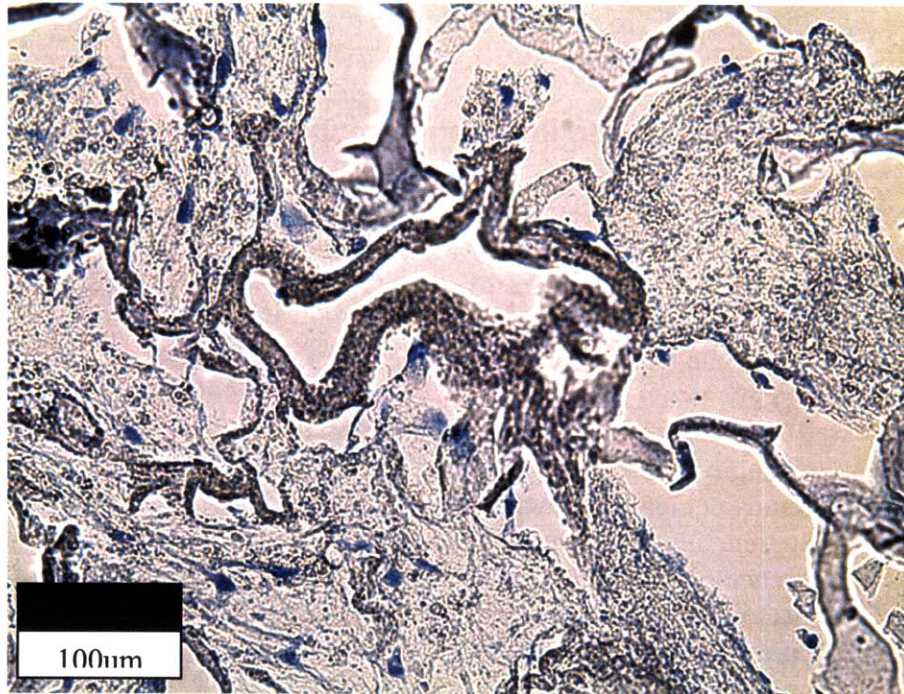
Fig 2.9 Bioreactor at 4 weeks, type I collagen immunohistochemistry.
Type I newly synthesized collagen is visible. Cells appear elongated and with a
fibroblast-like appearance.

Fig 2.10a. Bioreactor construct at 4 weeks, Safranin-O stain. Dense fibrous newly
synthesized matrix is apparent.
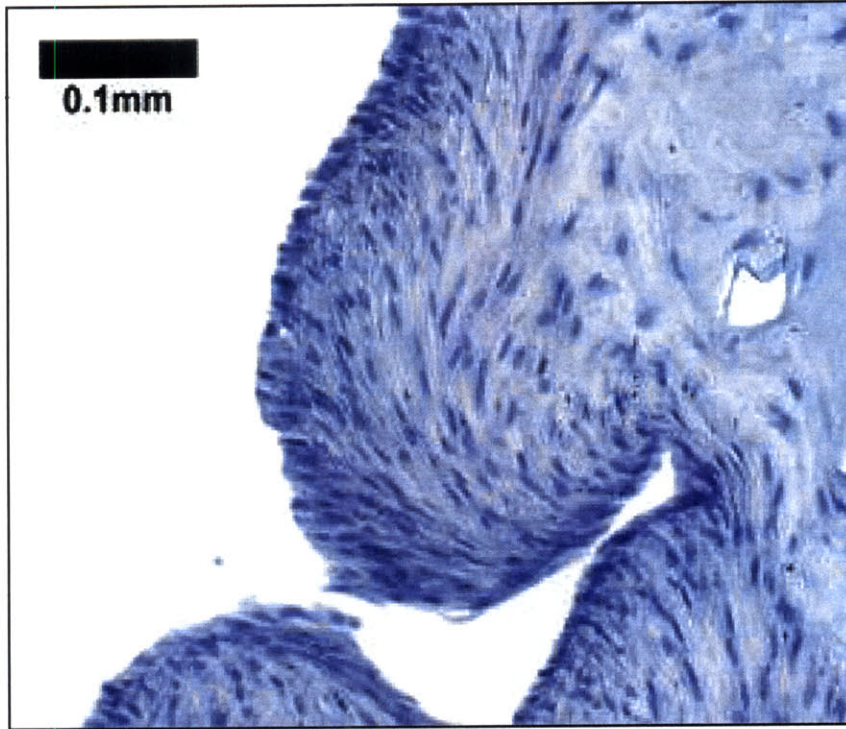
Fig 2.10b. Static construct at 4 weeks, Safranin-O stain. Fibrous tissue is also apparent on the edges of the scaffold
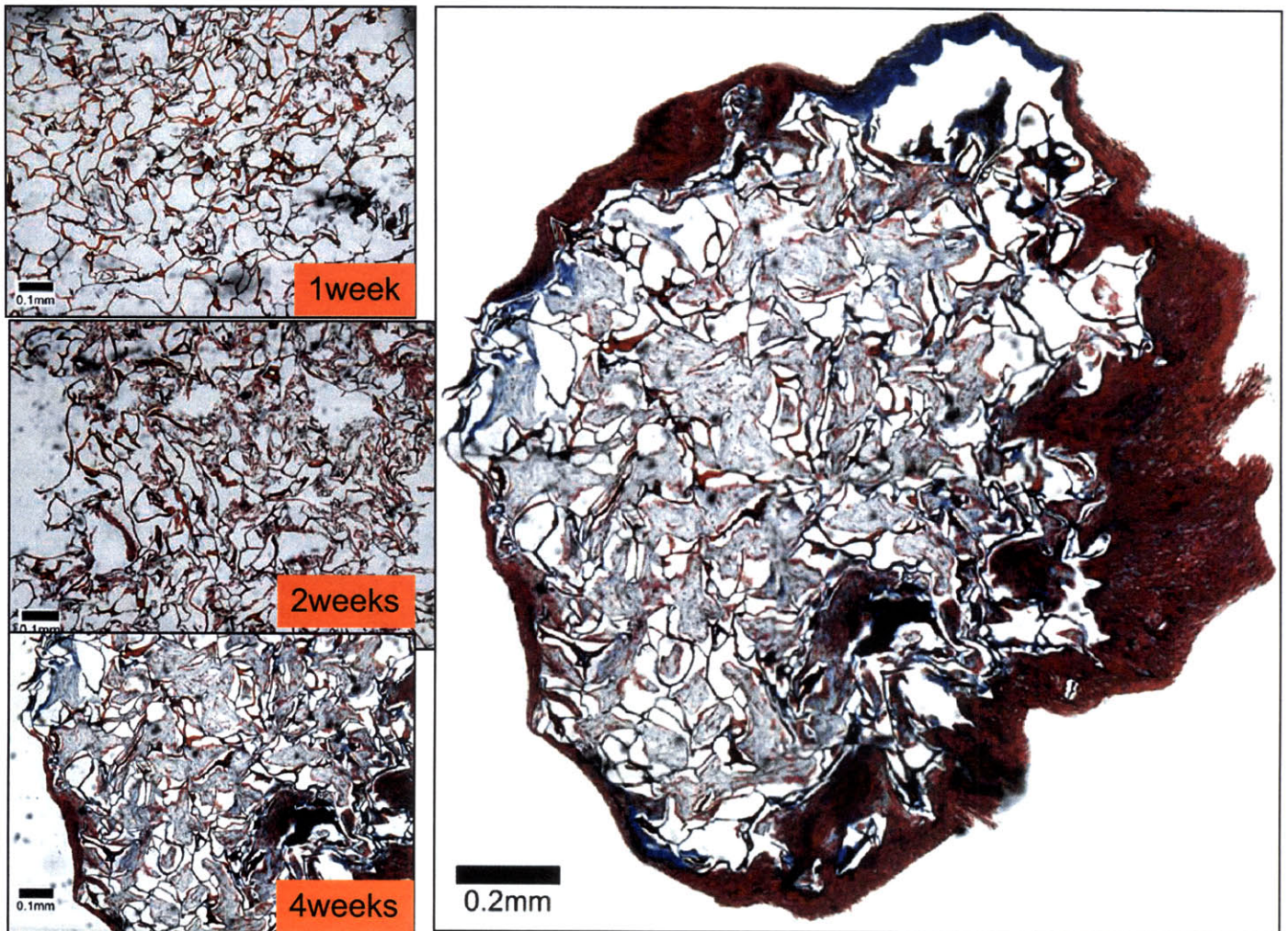
Fig 2.11 Bioreactor constructs evolution between 1 and 4 weeks. Newly synthesized matrix is apparent and uniform throughout the construct (Left). General appearance of a bioreactor construct after 4 weeks of culture. Dense fibrous tissue is apparent to the right side of the scaffold (Right).
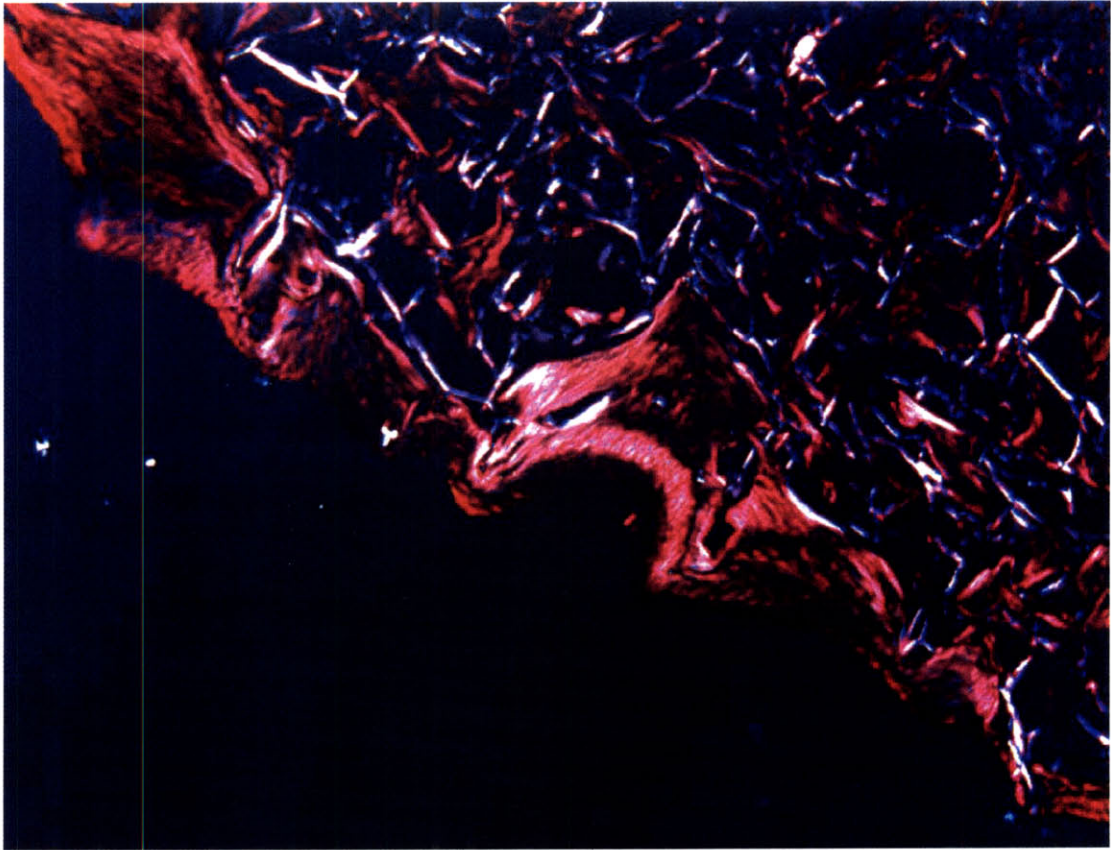
Fig.2.12 polarized light micrograph showing the orientation of the collagen fibers in the scaffold.

4. Discussion

The present study revealed advantages of a rotating wall bioreactor compared to static culture in favoring the development of tissue constructs from adult canine annulus fibrosus cell-seeded type II collagen-GAG scaffolds:

1) faster (by 2 weeks) formation of a cartilaginous construct with sufficient mechanical integrity to be handled and implanted;

2) increased proliferation of cells and mass of the constructs from 2 to 4 weeks of culture; and

3) increased biosynthetic rates and accumulated GAG over the 4-week time period of the study.

These three points confirm the three hypotheses that were made in the introduction of this chapter.

		Bioreactor Exp1	Controls Exp1	Bioreactor Exp2	Controls Exp2
General	number of cells (average during the first 2 weeks)	830,000	500,000	2,500,000	1,400,000
	Accumulated GAG % mass (at 4 weeks)				
Biosynthesis	Protein biosynthesis (average)	0.6	0.3	0.08	0.3
	GAG biosynthesis (average)	0.24	0.125	0.04	0.08

Fig.2.13. Summary of key results in the two bioreactor experiments

Future studies will be required to determine if the cartilaginous constructs produced in the bioreactor are capable of improving the reparative process in defects in the annulus fibrosus in animal models. Additional studies will also be required to determine if supplementation of static or dynamic cultures with other growth factors can result in constructs with compositions and properties closer to those of the annulus. After more than 2 weeks of culture, the cartilaginous appearance of the scaffolds cultured in the bioreactor was of importance in the perspective of an implant to repair annulus fibrosus defects. Although its mechanical properties would need to be evaluated, the general appearance was promising as well as the resistance to compression when pressed with forceps. Scaffolds, however, did not fully preserve their disc-like appearance, and after 4 weeks, they had slightly bent and looked like small cups. This was likely due to the flow of fluid inside the bioreactor and also verifies the mathematical modeling of tissue growth of another study [22]. This could prevent easy insertion in an animal model defect.

Although cells were uniformly distributed throughout the scaffolds in scaffolds cultured under static conditions, they appeared concentrated on the edges in the bioreactor samples, where the highest concentration of nutrients can be found. It would be preferable to have cells spread uniformly, by adopting a dynamic seeding method that would allow cells to uniformly occupy the pores of the scaffold. In both types of cultures, cell density within the scaffold did not appear very high: although 2 million cells had originally been seeded on each scaffold, the number of cells dropped to around 500,000 for 1% FBS samples and bioreactor samples, using a 3.6pg of DNA per cell as found in prior work. In 10% FBS samples, cell density was nearly preserved, with a number of cells of about 1.6 million per scaffold after 1 week. This higher number of cells in the

10% FBS scaffolds compared to the 1% FBS scaffolds was expected as FBS enhances cell proliferation in the scaffolds. A positive effect of the bioreactor cultures was an enhancement of cell proliferation, as cell number increased 2-fold after 4 weeks of culture, in opposite to the static groups that lost cells during the same time frame.

Not only were the scaffolds cultured in the bioreactor better in appearance and in cell proliferation, they were also superior to the scaffolds in culture due to their higher biosynthesis rates and higher accumulated GAG, two very important factors (Figs 1., 2., 3 and 4.). The general stimulating effect of the bioreactor culture compared to the static culture was very important, effect that increased over time, reaching a maximum at 4 weeks of 6 times the value of static constructs for the GAG synthesis, and 3.2 times for protein synthesis. Cells cultured in 1% FBS had higher biosynthesis rates than cells cultured in 10% FBS, as expected as FBS stimulates cell proliferation which reduced the biosynthesis activity of the cells. The fact that GAG biosynthesis increased significantly, although the amount of GAG retained in the scaffold was still very low. This shows that much GAG was lost to the medium. A possible solution to prevent this loss would be to embed the scaffolds in a gel, as was done by Buschmann *et al.* [23] who contained newly synthesized GAG of cells embedded in an agarose gel and observed less loss of GAG. Another important fact was that cells cultured in the scaffolds and cultured in the bioreactor continued to synthesize collagen type I molecules, although they stained negative for collagen type II. Annulus fibrosus cells do synthesize both collagen types although type I collagen makes up the majority of the annulus.

These globally positive results could be further improved by increasing gas exchange through the core membrane of the bioreactor. Indeed, gas exchange was done statically throughout the porous membrane, without the use of an additional pump. Vunjak *et al.* have shown that with chondrocytes, gas exchange was an essential component to further increase the tissue appearance and cell proliferation [24]. This should be explored in future studies.

It was of importance to note the experimental challenge of the experiments: when cell-seeded scaffolds were put in the bioreactor just a few hours after having been seeded, nearly all the cells were lost to the medium and never recovered. This explained why it was preferable to wait at least 3 days in the culture plates before placing the cells in the rotating environment. Another challenge was the reproducibility of the results, as sometimes, even if scaffolds had been cultured in the plates for a long enough period, cells were still lost to the medium.

The results of the present study parallel prior work [15] demonstrating that annulus fibrosus cells can be maintained in collagen-based scaffolds and can synthesize matrix molecules. That prior work underscored the challenge in formulating scaffolds and culture conditions that result in the increased synthesis and retention of these matrix molecules in the tissue engineered construct.

Conclusion: a rotating bioreactor enhanced the cell proliferation, increased the matrix synthesis, particularly of GAG, maintained the production of type I collagen. Future work

will try to identify the exact nature of the matrix, and try to find a way to obtain more repeatable results. The results of this study recommend the use of a rotating vessel as a bioreactor to stimulate the synthesis of the matrix by AF cells seeded in type II collagen-GAG scaffolds. Future work will also be required to compare the *in vivo* regenerative potential of such scaffolds with constructs cultured in other conditions, such as growth factor-supplemented medium.

Nota Bene: due to the low reproducibility of the experiments with loss of cells to the medium, the bioreactor was not used in the following studies of this thesis.

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CHAPTER 3 – EFFECTS OF GROWTH FACTORS: TGF-β1, FGF-2 AND OP-1

In this chapter, we evaluate the potential effect of a group of select growth factors on the cell proliferation and biosynthetic rates of the AF cells seeded in a collagen-GAG scaffold (the same scaffold as used in the previous chapter). The main goal of this new set of experiments was to find the optimal medium composition for the *in vitro* culture of these constructs in the perspective of future implantation in an animal model. Indeed, serum-supplemented medium encounters difficulties in being accepted by the U.S. Food and Drug Administration (FDA) due to the variations in the FBS source and content. Another goal was to see whether cells could thrive in this serum-free environment and if they could keep their phenotype.

The selected growth factors included transforming growth factor beta-1, TGF-β1, fibroblast growth factor-2, FGF-2 and the osteogenic protein-1, OP-1. These growth factors had demonstrated in prior work to be part of or to have potential benefit for the regeneration of damaged or degenerated intervertebral discs.

1. Introduction

a. Main challenges: serum, growth factor type/concentration, absence of markers, and effect of non essential nutrients

There are two main criteria used when identifying the most important growth factors. These growth factors should:
- improve proliferation and biosynthetic rates while maintaining phenotypic identity of the cells.
- prepare a well-defined medium composition that could be FDA approved, as serum supplemented media are not accepted by FDA

Previous studies ([1, 2]) have shown that IVD cells cannot thrive in a serum free environment without the addition of growth factors. Besides, a certain number of growth factors have been found in degenerating discs ([3-5]) and in painful but morphologically intact discs ([6, 7]). Cells receptors for these growth factors and/or mRNA expression of these molecules were also found on disc cells. These findings strongly suggested that certain growth factors or their inhibitors could play a key role in the regenerative process of the degenerating IVD ([8]) . It was suggested that the misbalance between the anabolic processes, often led by growth factors, and the catabolic processes, often led by matrix degrading enzymes, could be the major reason of tissue degeneration. This prompted the investigation of a suitable mix of growth factors.

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To evaluate growth factor effects, one needs to:

- identify the markers of the IVD cells in order to make sure that the phenotype of the cells has been maintained. This still remains to be done for the disc cells.
- identify the effect and dose responses of growth factors on the behavior of the cells and cells-seeded scaffolds.

Several studies have tried to identify the presence and analyze the role of a few growth factors in the degeneration of the intervertebral disc, showing that among others, TGF-β1 and FGF-2 have a regulatory role in the degeneration of the IVD [5, 9, 10]. After an investigation of injected these growth factors at the site of discectomy to evaluate the regenerative response [11] TGF-β1 was shown to have a beneficial effect in slowing down the degeneration of the IVD. TGF-β1 was also identified in herniated disc samples discs ([3-5]) and IVD cells seem to produce this growth factor on their own. Another strong effect of TGF-β1 was an increase in cell proliferation and proteoglycan synthesis, as well as a decrease in the matrix degrading enzyme MMP-2 ([2, 12])

The basic fibroblast growth factor, bFGF or FGF-2, which was found next to the small blood vessels in herniated tissue led researchers to think that angiogenesis could be one additional response of the disc to injuries ([4, 5, 13]).

These *in vivo* discoveries, however, do not enable to understand how cells behave *in vitro* in the presence of these growth factors, and this is the purpose of this research. To fill this gap, other studies have observed the increased proliferative and biosynthetic activity of other chondrocytic cell types (*viz.*, articular chondrocytes) when cultured in growth factor (GF)-supplemented medium. For IVD cells, stimulating effect of BMP-2 on bovine cells was reported [14], as well as the anti-apoptotic effect of IGF-1 and PDGF [15]. If positive effects of TGF-β1 and FGF-2 have been observed [1, 2] by nucleus pulposus cells, cultured in plates or three dimensional cultures such as agarose, alginate culture or pellet culture[2, 16], only little work has reported the use of AF cells in collagen-GAG matrices cultured in GF supplemented media. Alini *et al.* reported the beneficial effect of these 2 growth factors on IVD cells seeded on a collagen-hyaluronan scaffold [17].

OP-1, which has traditionally been used to induce bone synthesis, was directly injected in the disc resulting in a restoration of disc height, stable for up to eight weeks after injection ([18-21]) and in a decrease in pain in a rat model ([22]). This shows also the potential benefit of this growth factor to regenerate or maintain the disc properties.

All these prior results prompted the investigation of the behavior of AF cells cultured in type II collagen-chondroitin sulfate matrices in TGF-β1-, FGF-2-or OP-1 supplemented medium. TGF-β1 was chosen because it has often been reported to have a strong effect on metabolism and proliferation of chondrocytes and IVD cells [23], FGF-2 as its effect on AF cells cultured in plates was positive [1] and OP-1 due to its height restoration effect in the rat model mentioned above. A mix of TGF-β1 and FGF-2 was also explored.

Note: As in chapter 2, in this study, canine cells were used because of the possible future implantation of the constructs in a canine model, and their use in prior work [24, 25]. Scaffolds were cross-linked with a carbodiimide treatment as in chapter 2.

Specific hypotheses for this work are:
1. The above mentioned growth factors upregulate cell proliferation,
2. The above mentioned growth factors upregulate matrix biosynthesis rates,
3. The above mentioned growth factors have a strong effect in generating a more uniform matrix.

2. Materials and Methods

a. Experimental Design

Two experiments were conducted, the first one to evaluate the effects of TGF-β1 and FGF-2, the second one to compare OP-1 and a mix of TGF-β1 and FGF-2.

Cells from 6 adult dogs were cultured and seeded separately in type II collagen-GAG scaffolds. In a first experiment, cell-seeded constructs were then separated in 5 categories according to the medium (exact composition is described below) in which they were cultured:
   (1) medium supplemented with 1% FBS (control group),
   (2) serum free medium supplemented with 1ng/mL of TGF-β1,
   (3) serum free medium supplemented with 5ng/mL of TGF-β1,
   (4) serum free medium supplemented with 2ng/mL of FGF-2,
   (5) serum free medium supplemented with 10ng/mL of FGF-2.

In a second experiment, cell-seeded constructs were separated in 4 groups:
   (1) 10% FBS medium supplemented with OP-1 at 100ng/mL,
   (2) 10% FBS medium supplemented with OP-1 at 200ng/mL,
   (3) 10% FBS medium supplemented with a mix of TGF-β1 and FGF-2, both at 10ng/mL,
   (4) 10% FBS medium (control group).

The constructs were cultured for 1, 2 and 4 weeks (1st experiment) and 10,20 and 40 days (2nd experiment), and DNA content, accumulated GAG content, rates of total protein and GAG synthesis retained in the scaffolds were evaluated from the sacrificed samples (n=6, each sample from constructs seeded with cells from one of the 6 dogs). Histology and immunohistochemistry for type I and II collagens, were performed at each time point (n=2).

b. Scaffold Manufacturing and Cross-linking Treatment

Type II collagen-GAG matrices were produced by mixing porcine type II collagen (Chondrocell slurry, Wolhusen, Switzerland) and chondroitin 6-sulfate (Chondroitin

Sulfate, C-4384, from shark cartilage, Sigma Chemical, St. Louis, MO) at a concentration of 0.0105g per 20mL of collagen slurry. After 30 minutes of mixing, the blended slurry was poured in six well plates, 4mL per well and freeze-dried following a temperature ramping protocol from room temperature to -40°C in about 3hour [26]. Vacuum pressure was less than 200mTorr. Resulting pore diameter was evaluated to be 202±51μm, with a porosity of 91±2 and 6.7±2.2 % (w/w) of GAG content (mean ± standard deviation, SD). Freeze-dried sheets of the collagen matrices were approximately 3 mm thick.

The collagen sponge-like sheets were then cross-linked by dehydrothermal (DHT) treatment at 105°C for 24 hours. Nine mm diameter discs were punched from the sheets and the resulting samples were additionally cross-linked by immersion in 1-ethyl-3,3 dimethetylaminopropyl carbodiimide (EDAC; 6 mmol EDAC per gram of collagen, 5:2 EDAC/NHS ratio, in distilled sterile H2O) for 2 hours at room temperature. Matrices were then washed twice and kept in distilled water overnight at 4°C.

c. Cells and Seeding of Scaffolds

The AF cells were extracted from enzymatic digestion of the L1-L5 lumbar IVDs of 6 adult dogs (2-7 years old). Care was taken to exclude the nucleus pulposus and outer regions of the AF, which could clearly be distinguished in these canine IVDs. Enzymatic digestion was performed within 4-6 hours after harvest following a procedure detailed in [27], and cells from each of the 6 animals were expanded separately through 2 subcultures (i.e. to passage 2). Prior work [28] had demonstrated the phenotypic stability of 4th passage AF cells in two- and three-dimensional cultures, which justifies our use of passage 4 or lower AF cells for tissue engineering purposes.

Two million passage 2 AF cells were seeded onto the scaffolds, whose excess of water had been removed prior to seeding by blotting on sterile filter paper. One million cells in 25 μL of complete medium containing DMEM/F12 (#11320-033, Life Technologies, Grand Island, NY), 1% (5mL) of antibiotic-antimycotic solution (Gibco-BRL No. 15240-096, Life Technologies), 2% (10mL of 0.025g/L) of L-ascorbic acid phosphate (magnesium salt n-hydrate; #D13-12061) and 1% (5mL) of L-glutamine (#25030-81, Life Technologies), were pipetted onto one side of the constructs. After 10 minutes, another one million cells were pipetted onto the same side. The cell-seeded scaffolds were then placed onto 6-well plates previously coated with a 4% (w/v) agarose gel and incubated at 37°C in complete medium supplemented with 10% FBS (FBS, Hyclone Technologies, Logan, UT). After 3 days in culture, scaffolds were incubated in their respective complete medium, either with 1% FBS for the controls or in a serum-free environment for scaffolds cultured in a growth factor supplemented medium. This discrepancy between the first 3 days of culture was decided in order to facilitate cell attachment and proliferation during the first 3 days of culture.

Growth factors were used with the above mentioned doses for each group. TGF-β1 was ordered from R&D systems, Minneapolis. Bovine FGF-2 was from R&D

systems, Minneapolis. OP-1 was from Stryker Biotech. The controls were cultured in 1% FBS complete medium as recommended by previous studies [1, 2].

d. Radiolabel Incorporation

The rates of synthesis of proteoglycans and total protein retained in the scaffolds over a period of 24 hours were assayed with the use of radiolabeled proline (proline, L-[2,3,4,5-^{3}H]; #NET 483, Perkin Elmer, Boston, MA) and radioactive sulfate ^{35}S (#NET041H, Perkin Elmer). Cell-seeded samples were incubated in radiolabeled medium containing 10µCi/mL of each isotope. After a period of 24 hours in incubation, unincorporated sulfate was removed by rinsing 5 times 15minutes at 4°C with a solution of phosphate buffered saline supplemented with 0.8 mM Na_2SO_4 (anhydrous sodium sulfate; Sigma) and 1.0 mM L-proline (P8449, Sigma). The matrix discs were then frozen at -20°C, freeze-dried thoroughly and digested at 60°C in 0.125 mg of papain (Sigma #P3125) per 6.25 mg of dry sample. 100mL of the stock papain buffer solution was composed of 2.5 mL of 0.5M dibasic stock solution, 18 mL of 0.5M monobasic stock solution, 80 mL distilled water, 88 mg L(+) cysteine HCl, and 186 mg disodium ethylenediamenetetraacetate (EDTA, Fischer S-657), with the pH adjusted to 6.2 with a few drops of concentrated hydrochloric acid.

Radiolabel digests were assayed for radioactivity by combining 100µL of digest with 2mL of scintillation fluid (Scintiverse II, cat #SX12-4, Fisher Scientific, Fair Lawn NJ), and counted for 3 minutes in a liquid scintillation counter. Values of retained GAG and total protein synthesis rates were extrapolated from counts per minute using known amounts of radiolabeled medium. Mean counts per minute of unseeded control matrices was always negligible, showing the efficacy of the washing to remove all unincorporated isotopes. Therefore no adjustment of the results was required to account for the controls.

e. GAG Assay

To normalize the results, dry weight was measured after freeze-drying of the constructs. DNA quantification was performed using the Hoechst dye (#33258, Polyscience Inc., Northampton, UK) assay [29] from the papain digested samples, the amount of DNA being extrapolated from the standard curve of calf thymus DNA. Average amount of DNA content of unseeded scaffold was subtracted to each sample to obtain the net DNA content of each scaffold. Number of cells could be estimated using an amount of DNA of 3.6pg per canine AF cell, which had been measured by determining the DNA content of aliquots of known numbers of cells digested in papain following the same protocol.

f. DNA Assay

The total GAG content of the cell-seed constructs was determined using a dimethylene blue (DMMB) method [30]: A 100 µL aliquot of the papain digest was mixed with 2 ml

of the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The total amount of GAG was extrapolated from a standard curve of shark chondroitin sulfate. GAG content of unseeded constructs was also evaluated and subtracted from the total GAG content of the cell-seeded constructs to obtain the mass of newly synthesized accumulated GAG. This figure was then normalized by the dry mass of each construct to obtain the % mass of newly synthesized GAG.

g. Histological Evaluation

Selected samples of cell-seeded scaffolds were fixed in 10% buffered formalin for 3 days. Specimens were then processed in a tissue processor (Hypercenter XP, Shandon), and embedded in paraffin. Sections, 7 μm in thickness, were cut along the horizontal plane of the scaffolds. The microtomed sections were stained with hematoxylin and eosin, safranin-O (for proteoglycan), and Masson trichrome (for collagen). Slides were cover-slipped and sealed with cytoseal 60 cell mounting medium. The following features were examined by normal and polarized light microscopy: cell morphology, cell distribution, cell layers on the surface of the disc, collagen fiber organization, and GAG distribution.

Immunohistochemistry for type II collagen was performed on selected sections; previous studies had shown that the collagen type II of the matrices themselves did not stain positive if the matrices had been cross-linked with the EDAC. Deparaffinized and rehydrated sections were digested for 1 hour in 0.1% protease XIV, followed by blocking of non-specific binding with 5% horse serum. The primary antibody (CIIC1, mouse anti-chick type II collagen monoclonal antibody, Developmental Studies Hybridoma Bank, Iowa City, IA) was diluted 1:20 and applied for 1 hour. Negative controls were incubated with a universal mouse negative IgG (N1698, Dakocytomation, Carpinteria, CA) diluted to the same protein concentration, instead of the primary antibody. A biotinylated secondary antibody (horse anti-mouse IgG, Sigma) was applied for 45 minutes, followed by quenching of endogenous peroxidase with 3% hydrogen peroxide. Labeling was detected with an avidin-biotin complex (ABC kit, Vectastain, Vector), and diamenobenzadine (DAB, Vector). Counterstaining was performed using Harris hematoxylin for 2 minutes followed by 5 minutes wash.

h. Statistical Evaluation

Single- and multiple-factor analysis of variance (ANOVA) or Student t-tests were used to assess the effects of the duration of the culture and of type of culture on all of the measured variables. The Fisher least squares protected difference (LSPD) post-hoc test was also utilized for selected analyses using StatView (SAS Institute Inc., Cary, N.C.). All tests were run with the assumption of equal standard deviations. Data are presented with the error bars representing the standard error of the mean.

3. Results

a. Experiment 1 (1n/5ng TGF-β1, 2ng/10ng FGF-2)

Macroscopic Examination.

Prior studies where scaffolds were cultured in 10% FBS medium had shown that cell-seeded matrices rapidly changed their appearance within about 3 weeks, becoming white, shiny and resistant to forceps pressure, in contrary to non-cell-seeded scaffolds which remained fragile and sponge-like for long periods of culture. In this study, where scaffolds were cultured for 4 weeks, the 1% FBS controls and the scaffolds cultured in GF supplemented medium both did not change much their appearance and adopted a global appearance between the cartilaginous structure and the one of unseeded scaffolds.

Cell Proliferation

The average number of cells contained in each the different constructs was relatively equal for each different type of culture (Fig 3.1). However some differences can still be noted: Two-factor ANOVA revealed that there was a significant effect of the GF type on the number of cells per scaffold ($p<0.0001$), the maximum number of cells being reached with FGF-2 at the dose of 10ng/mL, with a number of about 700,000 cells at 4 weeks, 2.8 times fewer cells than was originally seeded onto the scaffold, but nearly twice as much as in the 1% FBS controls. In comparison, at 4 weeks, only 460,000 cells were present in 2ng/mL of FGF-2 scaffolds. There was no effect of the period of culture on the cell proliferation ($p>0.42$), although a general decreasing trend appeared in all groups except in the scaffolds cultured in FGF-2 at 10ng/mL which observed a continuous increase with time in its cell number. Fischer's post hoc test determined that there was no statistically significant difference between TGF-β1 cultured constructs and the controls ($p>0.24$), and that there was no large effect of the dose level between FGF-2 at 2ng/mL and 10ng/mL ($p=0.06$).

This larger amount of cells at 4 weeks in FGF-2 at 10ng/mL was also observed in the % of mass DNA, with a significantly higher percentage of mass DNA in this type of scaffold compared to other types which did not present any significant difference among each other.
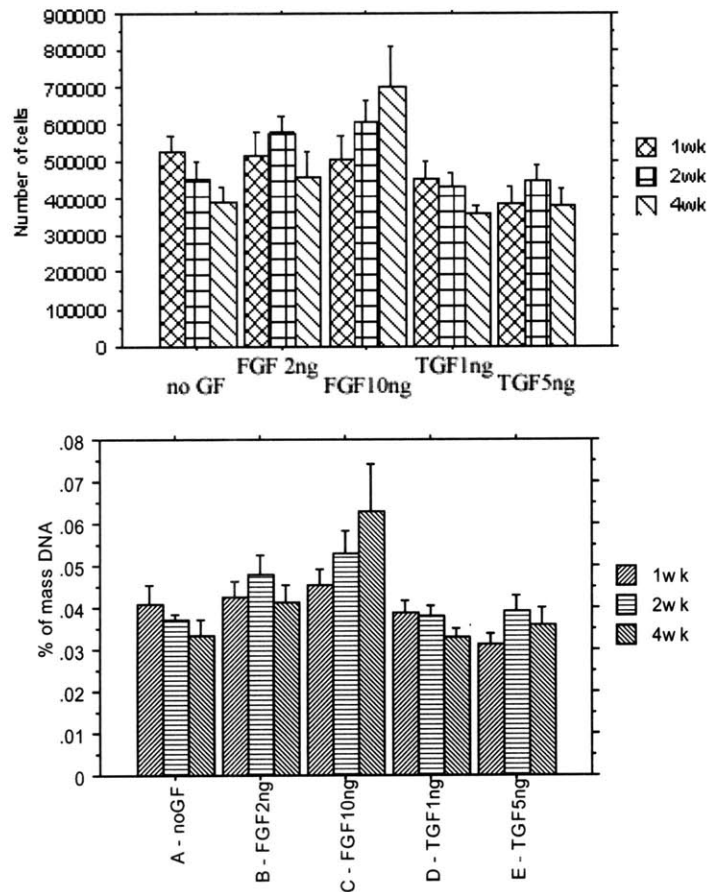
Fig 3.1.   Number of cells (a) and (b) % of dried mass that is DNA in the scaffolds cultured in A – 1% FBS, B – FGF-2 2ng/mL, C – FGF-2 10ng/mL, D – TGF-β1 1ng/mL, E – TGF-β1 5ng/mL, after the respective time periods in culture, using a conversion factor of 3.6 pg DNA/cell.

Rates of Biosynthetic Activity

The level of GAG synthesis was not significantly different among all groups except that the growth factor groups had significantly higher levels at 4 weeks than the negligible values of the controls (Post-hoc, $p < 0.0001$). No other differences among the groups were to be noted. The level of GAG synthesis remained constant during the first 2 weeks of culture (post-hoc test, $p > 0.11$) and decreased by a factor of 4 in all groups from 2 to 4 weeks ($p < 0.0001$). There was a significant effect of time (2-factor ANOVA, $p < 0.0001$; power=1), but there was no significant effect of the GF group on this behavior (Fig 3.2a).

Rates of protein synthesis continuously decreased with time for all the constructs ($p < 0.00001$), by about a factor of 2 between 1 and 2 weeks and by another factor of 2 between 2 and 4 weeks. Two-factor ANOVA revealed that there were significant effects of time and type of growth factor on protein biosynthesis ($p < 0.05$; power>0.7). TGF- β1 cultured constructs presented a significantly lower protein biosynthesis than controls

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cultured in 1% FBS (post-hoc test, p=0.02), with in average, values that were 30% lower than the controls. FGF-2 samples on the contrary generated an amount of protein that was equivalent to the controls. The FGF-2 cultured at 10ng/mL had a slightly higher average than the ones cultured in 2ng/mL. (Fig 3.2b)
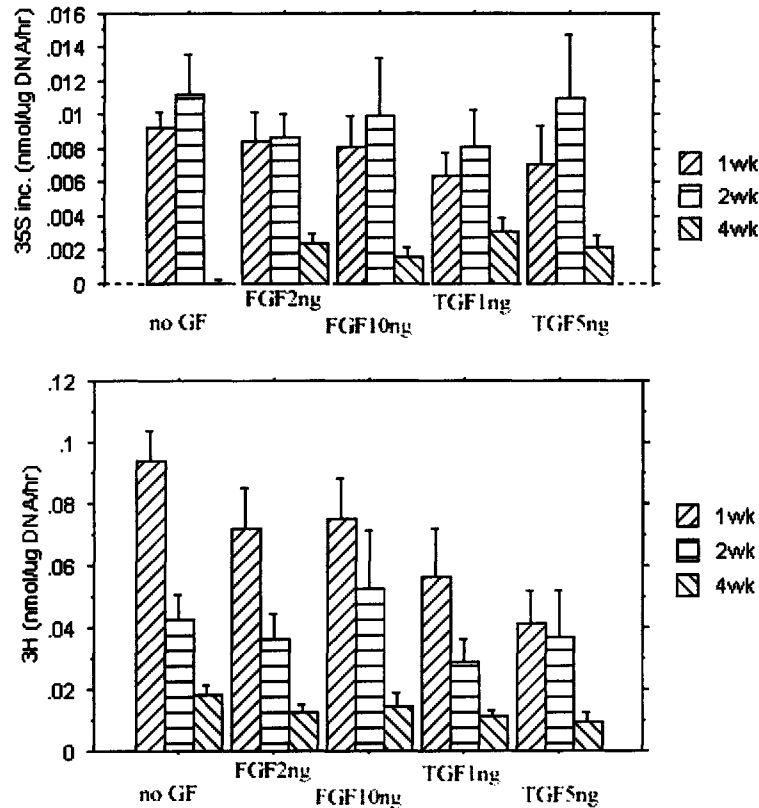
Fig 3.2.a (top) b.(bottom) - Rates of synthesis of (a) GAG and (b) protein. Normalized rates of (a) nmol ^{35}S incorporated per µg of DNA, per hour, and (b) nmol ^{3}H incorporated per µg of DNA, per hour.

Newly synthesized GAG (retained in the scaffold)

No important differences were notable in the amount of total GAG in the scaffolds. Measurement was made difficult as the original amount of GAG contained in the scaffolds from its production, was always much larger than the accumulated newly synthesized GAG.

At 1 week, the amount of newly synthesized GAG accumulated in the scaffolds was less than the original amount of GAG contained in the scaffolds before seeding resulting in negative values in the amount of accumulated GAG (Fig 3.3a). This could come from the fact that as cells contract the scaffold, GAG originally present in the matrices is lost into the medium, phenomenon which would not be observable for unseeded scaffolds, which do not contract.

After 2 and 4 weeks however, cells had generated enough GAG in all the groups to make up for the original loss of GAG. The controls group at 1% FBS and the FGF-2

groups presented the highest mass and % mass of accumulated GAG, with no statistically significant difference between them (Fig 3.3b). TGF-β1 samples had a lower amount of accumulated GAG resulting in an inferior scaffold at 4 weeks. This was confirmed by safranin-O histology of the constructs that showed muss less newly synthesized GAG in these constructs than in all other samples after 4 weeks.
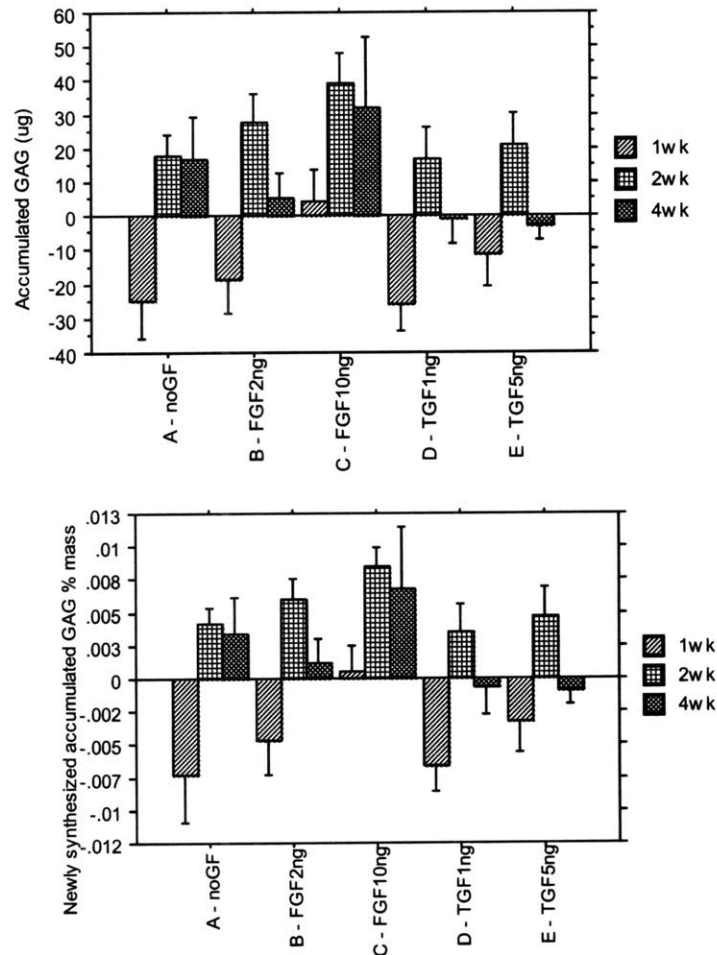
Fig 3.3.a (top) and b (bottom) – Accumulated GAG in the different groups

Histology and Immunohistochemistry

Histology revealed that cells were distributed throughout the collagen-GAG matrices in all the groups (Fig. 3.4 to 3.7). With increasing time in culture there was an apparent increase in the amount of newly synthesized extracellular matrix in the FGF-2 and control groups. In the TGF-β1 group, this amount of newly synthesized matrix was less significant than in the other groups. This matrix appeared as thin filamentous material. Cells could be found suspended from these filamentous cables (Fig. 3.4) as well as attached to the walls of the scaffolds. Cells appeared in elongated and stellate morphologies: At 1 week, cells cultured in TGF-β1 were mostly elongated and high cell density areas had started making up new matrix (Fig 3.4). In FGF-2 constructs, cells were mostly rounded with a lower amount of newly synthesized matrix (Fig 3.5). In the

controls group, half of the population of cells was rounded and half of the population was elongated. Again, in high density areas, cells had started making up new matrix. After 4 weeks, TGF- β1 constructs had significantly fewer cells than in the other groups (Fig 3.6). The amount of newly synthesized matrix also appeared lower compared to the FGF-2 and controls groups. In the FGF-2 group, cells were now more elongated and there were more cells than at 1 week (Fig 3.7). Control groups looked similar to FGF-2 constructs with a lower amount of newly synthesized matrix.

Safranin-O staining revealed that GAG was principally located next to the sides of the matrices mostly surrounding cells.

Of note was the formation of tissue-like structure in the scaffolds in most groups. In some cases this tissue structure appeared to be displacing the collagen-GAG scaffold. Collagen type II immunohistochemistry was negative, indicating that cells were not producing collagen type II collagen in any of the groups.
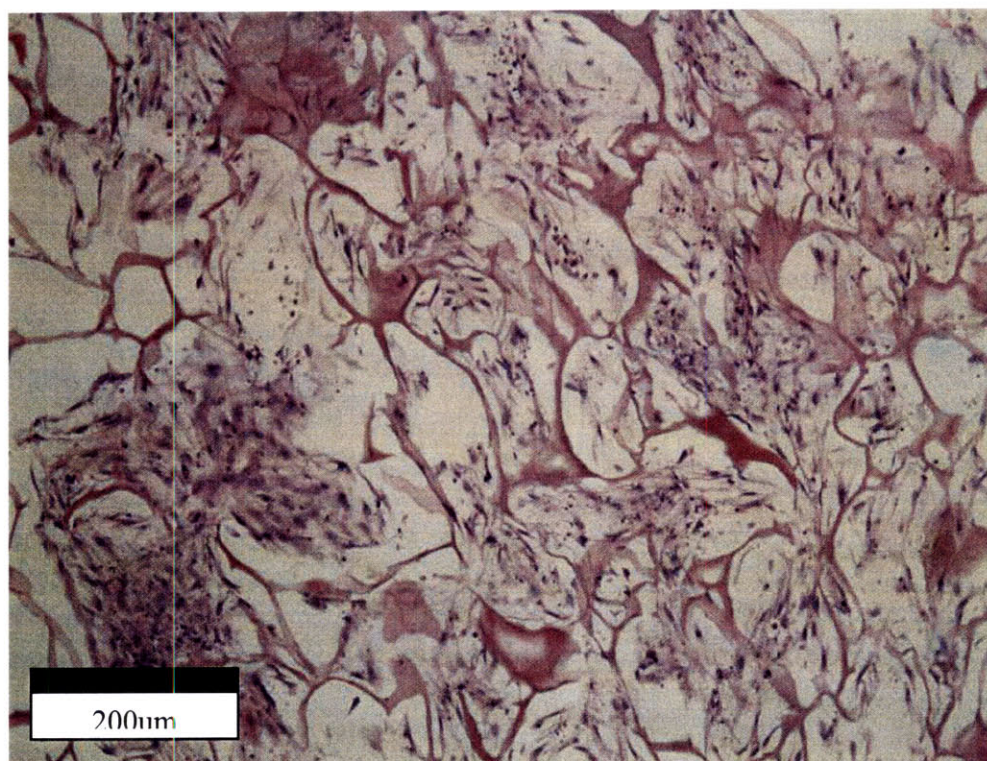
Fig 3.4. Micrograph of a cell-seeded collagen-GAG scaffold cultured for 1 week in TGF-β1 at 5ng/mL (10x)
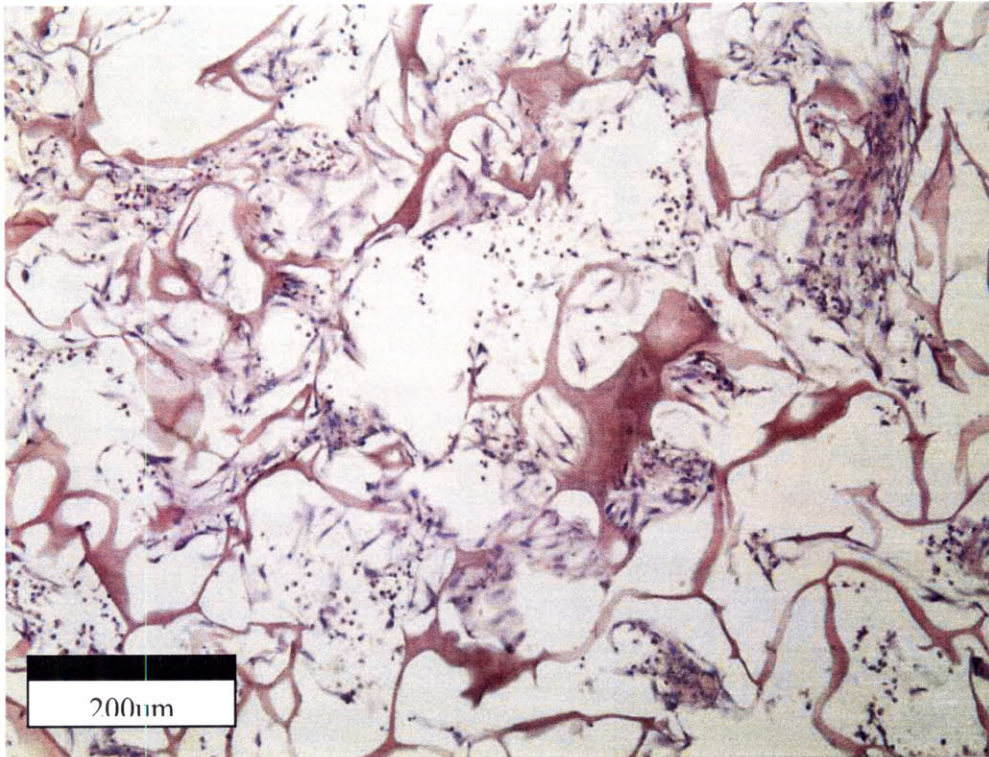
Fig 3.5. Micrograph of a cell-seeded collagen-GAG scaffold cultured for 1 week in FGF-2 at 10ng/mL (10x)

Fig 3.6. Micrograph of a cell-seeded collagen-GAG scaffold cultured for 4 weeks in TGF- $\beta1$ at 5ng/mL (20x)
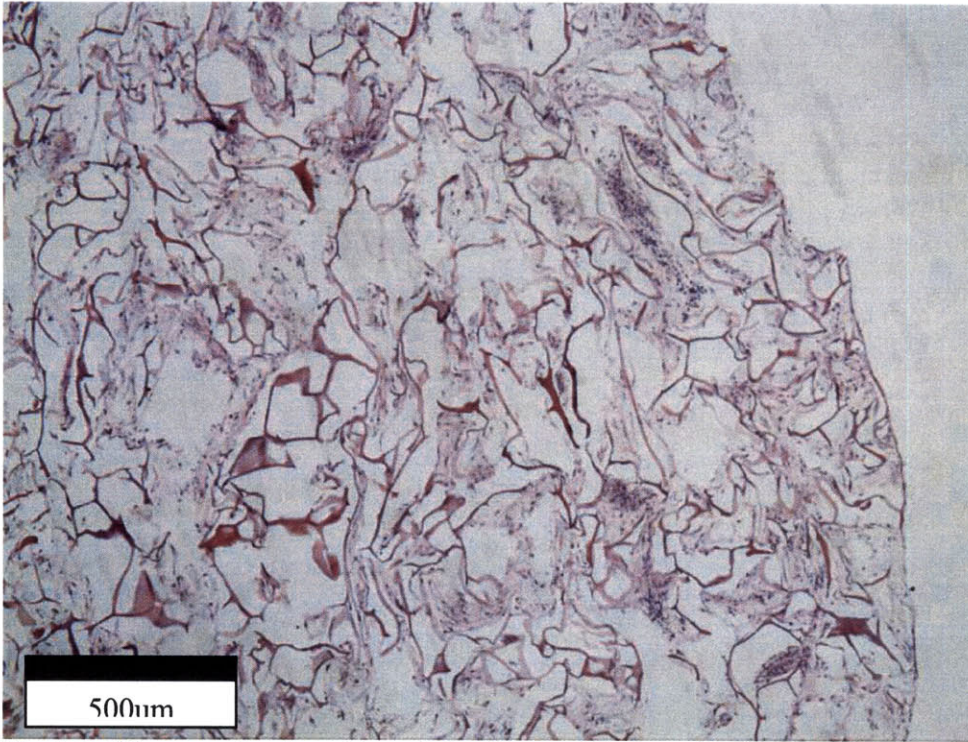
Fig 3.7. Micrograph of a cell-seeded collagen-GAG scaffold cultured for 4 weeks in FGF-2 at 10ng/mL (4x)

b. Experiment 2 (100ng/200ng OP-1 and TGF-β1/FGF-2 both at 10ng/mL)

Macroscopic Examination.

Samples looked similar to what was observed in the first experiment.

Dry Mass

Whereas dry mass of the constructs remained constant over the time of the experiment in the controls and in the TGF/FGF group, dry mass increased continuously from 10 to 40 days (by 20%) in the OP-1 constructs (ANOVA, $p < 0.0001$).

Cell Proliferation

Samples cultured in OP-1 had a constant DNA content over time, whereas the DNA content of the control and TGF/FGF groups decreased from 10 to 40 days (ANOVA, $p < 0.07$) (Fig. 3.8). At 40 days, the DNA content of the OP-1 group was more than 30% higher than that of the control and TGF/FGF groups. The % of mass DNA decreased in all samples from 10 to 40 days although OP-1 samples after 40 days still had a significantly higher % mass DNA than the controls. There was no significant difference between TGF/FGF constructs and the controls.
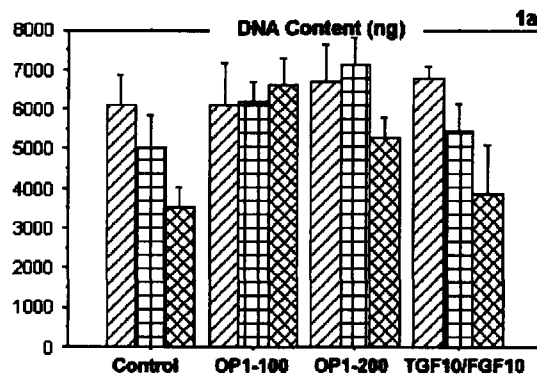
Fig 3.8 – DNA content (ng) in the 4 groups of cell-seeded scaffolds cultured for 10, 20 and 40 days. n=6; mean±SEM.

Newly synthesized GAG (retained in the scaffold)

At 10 and 20 days, the amount of accumulated GAG was very similar in all samples. At 40 days, however, this amount was nearly 2.5 times higher in the samples with OP-1 at 100ng/mL than in the control group (Fig 3.9). A positive effect of TGF/FGF and OP-1 was observed on the % GAG mass with the largest effect coming from a 2-fold increase for samples in OP-1 at 100ng/mL. When normalized by the mass of DNA, the

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accumulated GAG retained in the scaffold increased with time but was not statistically significant among all the groups.
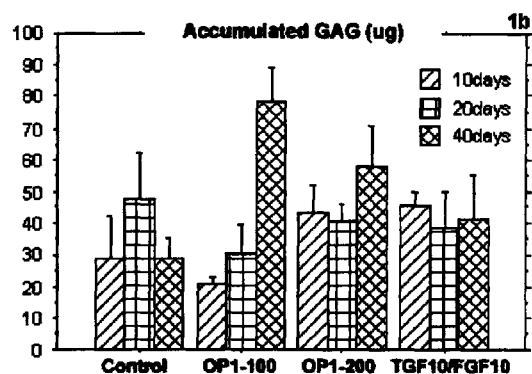
Fig 3.9 – Accumulated GAG content (ug) in the 4 groups of cell-seeded scaffolds cultured for 10, 20 and 40 days. n=6; mean±SEM.

Rates of Biosynthetic Activity

There was no significant effect of the type of culture on the GAG synthesis rate (unpaired t-test, $p > 0.87$) (Fig 3.10). The rate of protein incorporation increased significantly between 20 and 40 days (Fig 3.11) with the highest increases in the OP-1 at 200ng/mL and in the TGF/FGF groups, the 2 other groups' levels remaining constant over time.
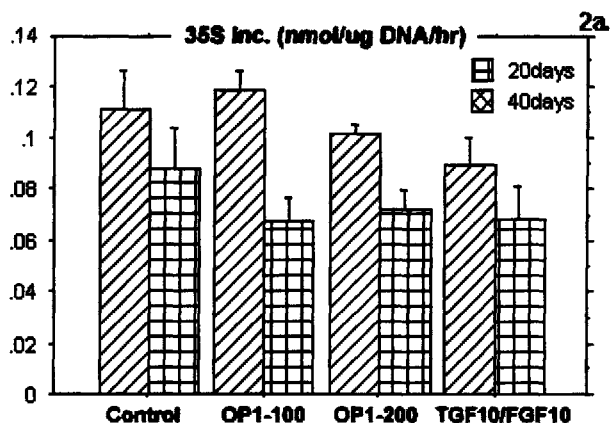
Fig 3.10 – Proteoglycans synthesized and retained in the scaffold during a period of 24 hours and normalized by the amount of DNA and per hour. n=6; mean±SEM.

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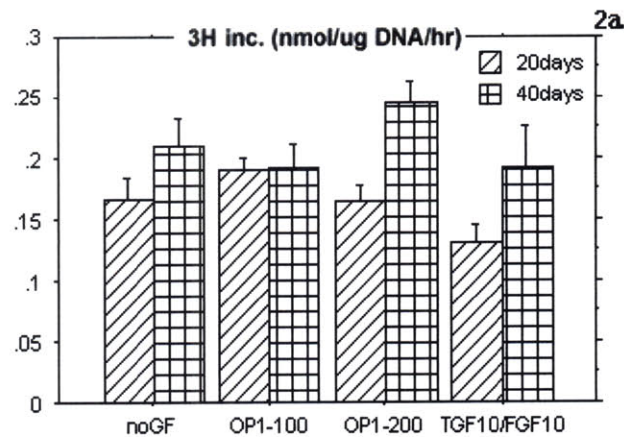
Fig 3.11 – Total proteins synthesized and retained in the scaffold during a period of 24 hours and normalized by the amount of DNA and per hour. n=6; mean±SEM.

Histology and Immunohistochemistry

Histological results showed that most of the newly synthesized tissue, which had the appearance of a fibrocollagenous matrix, was located on the edges of the scaffolds. Fibroblastic cells on the edges were elongated and those in the center of the matrix were rounded. GAG mostly surrounded the cells. There was more visible newly synthesized tissue at 40 days (Fig. 3.12) than at 20 days in most scaffolds, the densest new matrix being obtained with scaffolds grown in GF-supplemented medium.
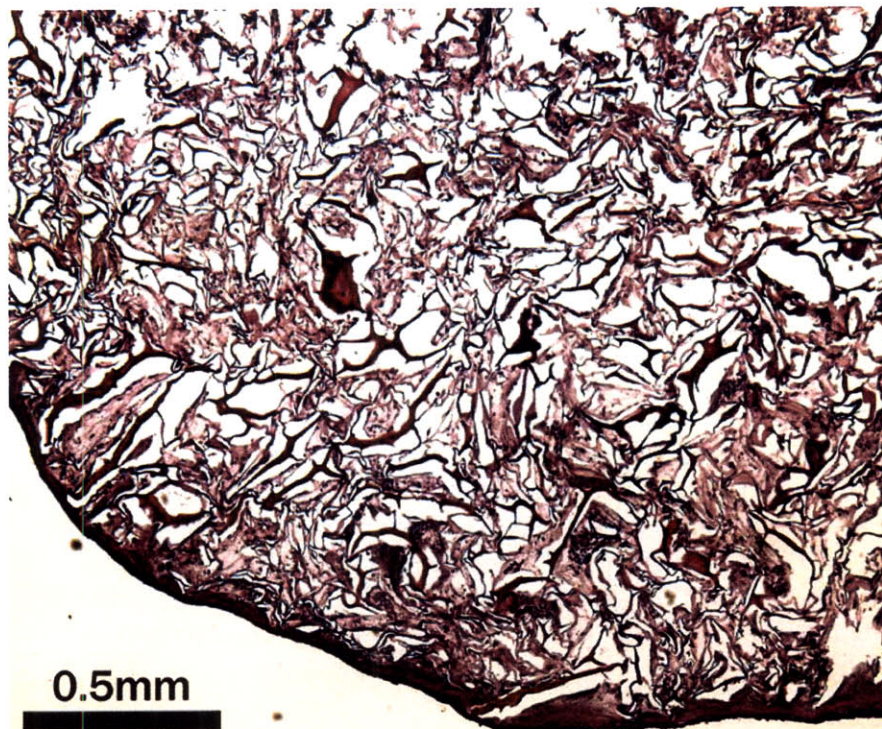
Fig 3.12. H&E staining of a scaffold cultured in OP-1 at 100ng/mL for 40 days.

4. Discussion

It was of interest that in both experiments, the growth factors maintained the biosynthesis activity of the cells during the time of the culture.

Experiment 1
The most notable effect was the absence of significant difference between the FGF-2 at 2 or 10ng/mL and the controls on both the proteoglycan and the protein biosynthesis. On the other hand, TGF- β1 supplemented media had less effect (30% less) on the biosynthesis than the controls or the FGF-2 samples. If other studies had shown the beneficial effect of TGF- β1 as a regulatory role in the IVD degeneration process or in the culture of monolayer or 3D culture IVD cells seeded scaffolds, this effect did not appear evident in our results.

Besides, the positive effect of FGF-2 was observed in the amount of accumulated GAG in the scaffold, where FGF-2 samples accumulated the same amount of GAG per unit mass as the controls group. In the opposite, in accordance with the biosynthesis trends, TGF- β1 samples retained a lower amount of GAG per unit mass. Moreover, the total amount of GAG retained in TGF- β1 samples was negligible even after 4 weeks of culture. As GAG synthesis rates were comparable for all construct types, these results show that newly synthesized GAG found a more suitable environment to stay within the matrix in FGF-2 and 1% FBS supplemented medium. Of significant note was the fact that in all scaffolds, this amount of accumulated GAG per unit mass decreased significantly from 2 to 4 weeks: in the perspective of implanting the scaffold in an animal model, it would be recommended to implant the cell-seeded scaffold after 2 weeks of culture.

Cell proliferation, reflected in the quantity of DNA in the scaffolds, was the highest after 4 weeks for FGF-2 samples at 10ng/mL, with a number of cells twice that in the controls group. Contrary to other studies, TGF- β1 did not stimulate cell proliferation. An explanation of this discrepancy could come from the fact that TGF- β1 has been shown to have relatively little effect for cells that are fully differentiated. If cell proliferation, in average of 500,000 cells per construct, seemed to be much lower than the initial seeding of 2 million cells per construct, there are different possible reasons:

- seeding technique may have been suboptimal: the pipeting technique is showing some of its limits in terms of accuracy although it has shown to have the best yield for initial cell attachment. In this work, constructs were seeded onto one side only, and it is unclear how deep the cells initially penetrated into the scaffold after being pipetted onto the surface of the matrix.
- Cells were placed in an environment free of FBS (except for the controls with 1% FBS) and it was expected that there would be fewer cells than in 10% FBS cultures. The goal of this study was more to evaluate maximal the biosynthetic activity induced by the presence of the GF in the medium rather than the proliferation ability of the cells, which has already been proven in numerous other studies [27].

		TGF-b1		FGF-2		Controls
		1ng/mL	5ng/mL	2ng/mL	10ng/mL	
General	number of cells (average)	400,000	400,000	500,000	600,000	450,000
	Accumulated GAG % mass (at 4 weeks)	0%	0%	0.1%	0.7%	0.3%
Biosynthesis	Protein biosynthesis (average)	0.3	0.3	0.4	0.5	0.5
	GAG biosynthesis (average)	3.0E-4	3.4E-4	3.3E-4	3.6E-4	3.4E-4
Histology	New matrix synthesis (1wk)	+	+	0	0	+
	New matrix synthesis (4wk)	++	++	++++	++++	+++
	Cells shape (1wk)	elongated	elongated	rounded	rounded	rounded/elongated
	Cells shape (4wk)	elongated	elongated	elongated	elongated	rounded/elongated
	Cells density	high	high	high	high	high
	Cells density (4wk)	low	low	high	high	medium
	Type II immuno.	-	-	-	-	-

Fig 3.13. Summary table of key results – Experiment 1

In histological sections, it appeared that FGF-2 constructs contained more synthesized matrix than in the controls. This stimulus of FGF-2 on protein synthesis is new compared to other studies that did not report any increase, although these dealt with explants and not with cell seeded scaffolds. This prompts for further investigation on the effect of GF on the culture of AF cells seeded in collagen-GAG matrices. Furthermore, as the time in culture increased, accumulated GAG increased significantly which is of importance in the perspective of implanting the scaffold *in vivo*. The *in vivo* / *in vitro* comparison will also be made more interesting, considering that in the *in vivo* confined environment, newly synthesized proteins or GAG will not be lost in the medium as was observed in *in vivo* studies.

Experiment 2

Notable findings of this study were that OP-1 treatment resulted in maintenance of AF cell number in the scaffolds over the 40-day culture period and in an increased (2.5-fold) accumulation of GAG, particularly at the 100ng/mL dose compared to controls. The greater amount of accumulated GAG was likely associated with the increased cell number because there was no significant difference in the biosynthesis rates per DNA among the groups. The combination of FGF-2 and TGF-β1 failed to alter the behavior of the cells compared to the controls. The results of experiment 1 showed that FGF-2 and TGF-β1 supplementation of the medium separately promoted GAG and protein synthesis rates, compared to controls cultured in 1% FBS. The fact that the current study used 10% FBS, as has been previously reported [31], may explain the absence of an effect of the TGF-β1/FGF-2 combination in this particular experiment.

Of note also was that the ratio of new GAG/DNA was constant among the groups although this figure increased slightly with time in culture. This suggested that the cells in the various culture environments produced the same amount of GAG per cell. This result is consistent with the ^{35}S-sulfate incorporation data, which showed comparable values for all the groups.

		OP-1		TGF-b1 / FGF-2	Controls (10%FBS)
		100ng/mL	200ng/mL		
General	number of cells (average)	1.6M	1.7M	1.4M	1.3M
	Accumulated GAG mass (at 40 days)	75	55	40	30ug
Biosynthesis	Protein biosynthesis (average)	0.19	0.21	0.15	0.19
	GAG biosynthesis (average)	0.09	0.085	0.07	0.1

Fig 3.14. Summary table of key results – Experiment 2

5. Conclusion

In the introduction, we drew 3 hypotheses that this research had to verify:
1. The above mentioned growth factors upregulate cell proliferation,
2. The above mentioned growth factors upregulate matrix biosynthesis rates,
3. The above mentioned growth factors have a strong effect in generating a more uniform matrix.

We can confirm that FGF-2 and OP-1 upregulate cell proliferation and matrix biosynthesis. This result was less obvious for TGF-β1. Matrix uniformity in all the groups with cells more uniformly spread out than in the controls or previous in vitro experiments ([27]).

The results of these two experiments suggest that OP-1, FGF-2 or the mix TGF-β1 and FGF-2 could be ideal supplements to upregulate matrix synthesis *in vitro* and may have beneficial effects in stimulating matrix production by AF cells in the context of an implant for IVD tissue engineering.

Additional work will be required to identify optimal culture conditions and GF doses. Other possible GF to consider for further investigation are the insulin-growth factor.

Chapter 4 will continue this thesis by evaluating the effect of scaffold make up and handling processes. For this perspective, we will choose the mix TGF-β1 and FGF-2 for the rest of this study.

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CHAPTER 4 – EFFECTS OF CROSS-LINKING AND SCAFFOLD COMPOSITION: EDC VS. DHT, COLLAGEN GAG VS. COLLAGEN ONLY.

The composition and cross-linking technique of a scaffold have been shown to influence importantly the behavior of cells seeded and cultured in these constructs. In this chapter, we evaluate (1) the effects of two widely used cross-linking methods, dehydrothermal treatment (DHT) and carbodiimide (EDAC) as well as: (2) the effect of the presence of GAG in the type II collagen scaffolds; (3) the effect of FBS; and (4) the effect of selected growth factors. Serum-free medium supplemented with a mix of TGF-β1 and FGF-2 was used for the culture of the cell-seeded scaffolds.

The main goal of these experiments was to find the scaffold make-up and handling techniques that will yield the optimal construct for implantation in the shortest amount of time, in the perspective of an animal trial. Adult goat cells were used in anticipation of the *in vivo* study to be performed in a goat model (chapter 5). The goat model was selected over the dog model which had previously been employed, on the basis of availability, and financial considerations. A comparison of caprine and canine cells was also made.

1. Background on scaffold composition, handling and treatment

a. Main challenges

The success of tissue engineering is strongly linked to the knowledge and understanding of scaffold properties and how these properties will affect cells behavior or the interaction with the host tissue. Extensive reviews can be found in *Principles of Tissue Engineering*, or *Practice of Tissue Engineering*, Langer and Vacanti *et al.* Numerous types of synthetic and naturally occurring polymers have been tried in various tissue engineering applications. Evaluation of the interaction between these scaffolds and the cells is often difficult to be quantified *in vivo*, requiring the design and experimentations of *in vitro* studies. Although these studies are far from completely replicating the *in vivo* environment, they still offer a good alternative to study cell adhesion, migration, differentiation, viability, biosynthetic behaviors and the changes in these variables with time. Scaffolds also enable the tissue engineer to evaluate the synthesized tissue and to hypothesize about how this tissue would interact with the host tissue.

Different types of synthetic polymers were used, some of them biodegradable or with adsorbed proteins to facilitate cell attachment, migration and growth. Surface morphology was also studied to control cell behavior. Finally, scaffold supplementation with genes has recently appeared.

Other types of polymer scaffolds have been used in original ways to provide a structure for cell proliferation and function: hydrogels, foams, fiber meshes, agarose gels are among the most widely studied.

In this thesis, we focus entirely on collagen-based scaffolds on the basis that their chemical make-up replicates certain features of the ECM of the tissue to be regeneratied and should help in cell adhesion, migration and function. Besides, degradation products of collagen are entirely non toxic.

Scaffold manufacturing processes are various and include fiber bonding, solvent casting and particulate leaching, membrane lamination, melt molding, extrusion, 3D printing, gas foaming, phase separation, polymer/ceramic composite foams, *in situ* polymerization and finally, freeze drying (*Principles of Tissue Engineering*, chap. 21). Freeze drying is the process that we selected throughout this thesis. It was selected based on 20 years of research and experience at the Fibers and Polymers Llaboratory at MIT and has yielded convincing results.

b. Scaffold composition

i. Collagen

Collagen is the major protein in the body with around 30% of the total proteins. Collagen is very abundant in tissues that require strength and flexibility, which is exactly the case of the intervertebral disc. Besides, collagen molecules contain ligands for the integrins of certain cell types that will facilitate cell attachment and migration and provide an *in vitro* environment similar to the native tissue. These integrins also help cell differentiation and biosynthesis. For example, fibroblasts grown on collagen scaffolds showed biosynthetic behavior that was very similar to the native tissue (Silver and Pins 1992). Chondrocytes, whose extracellular matrix main collagen type is type II also and retained their phenotype on collagen scaffolds (Toolan et al, 1996). The work of Yannas, *et al.*, also led to the creation of a regeneration template for dermis, confirming the promise of collagen matrices for the regeneration of an array of tissues, including intervertebral discs.

Recent studies ([1]) have shown that human disc cells cultured in a collagen sponge or in agarose were producing superior constructs than disc cells seeded in collagen gel or alginate.

As the intervertebral disc is composed mainly of type I and type II collagens, a collagen based scaffold, made of either of these two collagens types, could be a good start for tissue engineering of the intervertebral disc.

ii. GAG

GAGs are a major constituent of the intervertebral disc as seen in chapter 1. One of their roles is to preserve the hydration level of the disc, mostly of the nucleus, which is

necessary for the mechanical integrity of the disc. While the presence of GAG was proven useful in several collagen-based scaffolds ([2-6]), success was also observed with collagen-only-based scaffolds ([7]). The GAG that is the most widely used in the medical industry is hyaluronic acid (also called hyaluronan). Thanks to its length, it has a high water binding capacity and can easily be isolated. In the work of Alini *et al.*, hyaluronic acid was chosen to supplement a collagen scaffold for the regeneration of the IVD ([8, 9]). Other GAGs include chondroitin-sulfate ([10, 11]), dermatan sulfate, keratan sulfate and heparan sulfate, but few of these were used to study the regeneration of the IVD.

It thus appears necessary to evaluate the effect of the presence of GAG as a supplement of the collagen scaffold. The GAG that was chosen is chondroitin sulfate due to the prior studies that were made in our lab using this GAG and the intervertebral disc ([11]).

c. Cross-linking treatments

The advantage of using collagen scaffolds is that the tissues will treat the scaffold as if it was part of the tissue itself, which helps its integration. However, cross-linking the scaffold is necessary in order to: slow down the degradation of the collagen scaffolds; improve the mechanical properties of the scaffold; and enable more histogenesis. Cross-linking treatment of the scaffold will produce a stiffer and more slowly degradable scaffold. Both of these characteristics are important in the case of the IVD, in a mechanically challenging environment where there is also a slow turnover. Cross-linking is also often part of the sterilization process. At the same time, it is important not to use a too aggressive cross-linking treatment that would alter the functional properties of the collagen. The following paragraphs will review different cross-linking methods.

Glutaraldehyde treatment was originally the favored treatment for its ease and for its beneficial effect on mechanical properties, for its sterilizing properties and for the nearly undegradable scaffold that was obtained. However, certain experiments reported higher calcification *in vivo* of these cross-linked scaffolds in certain applications as well as a high toxicity of the degradation byproducts (*viz.*, the toxic glutaraldehyde molecule is released as the scaffold degrades). Moreover, the limited ability of the scaffold to remodel also reduced the attraction of glutaraldehyde as a cross-linking technique.

Carbodiimides (also called EDAC or EDC) have been used with high success: no toxic byproducts and good mechanical properties are often obtained. However, carbodiimide treated scaffolds which are highly cross-linked may have a problem of integration in the host tissue, and as a result become encapsulated by scar tissue instead of being integrated. In our case, studies presented in previous chapters used EDC cross-linked scaffold. In the perspective of an *in vivo* implant, it could appear necessary to use a less aggressive cross-linking method.

Dehydrothermal treatment (DHT) uses dehydration and thermal methods to bond the amino and carboxyl groups of the collagen fibrils. DHT-treated scaffolds are often

preferred when tissue and cellular ingrowth are desirable. The only drawbacks of DHT treatment is the weak mechanical properties as well as low rehydration capacity due to excessive dehydration due to denaturation of the collagen fibers.

Other cross-linking methods include the use of ultraviolet radiation and a polyepoxy compound. None of these were studied in this thesis.

In this study, we evaluate the difference between EDAC and DHT-only cross-linked scaffolds

d. Seeding techniques

There are very few published studies which systematically compare seeding techniques ([12-17]). However, the way that cells are seeded on the collagen construct will have a strong impact on the final aspect of the construct, as cell seeding techniques impact cell attachment yield and uniformity.

What is desired in our case is a uniform cell spreading throughout the scaffold and a high attachment yield so as to minimize the required amount of cells.

Controllable variables are the following:
- seed on wet or dry scaffold: chapters 2 and 3 seeded on a pre-wet scaffold that had been partially dried on filter paper. Although this technique had been traditionally used in our lab, we discovered that seeding on dry scaffolds could not only be much simpler and controllable, the amount of partial dehydration being very difficult to measure, but would also yield better results.
- use of dynamic methods or just static: a simpler dynamic method would consist in placing the scaffold in a small container filled with cells and medium and to actuate the container in order to let the cells attach to the scaffold. This approach was not used in this study.
- use injection methods: cells can be injected inside the scaffold with the help of a needle. This was done in the past for cells of larger dimensions (MSC cells for example)
- seeding density: this parameter was partly explored in this study and showed that densities between 50 and 100 million cells per mL did not result in significant changes in the resulting tissue.
- Amount of liquid seeded on the scaffold: knowing what amount of liquid (cells+medium) to seed is extremely important: too much liquid would result in a cell suspension to flow out of the scaffold due to saturation of the collagen sponge. Seeding too little would lead to poor spreading of the cells in the scaffold. Optimal volume required was evaluated empirically by taking scaffolds and seeding them with different amounts of liquids until saturation of the collagen sponge.
- seed on one side or both sides of the scaffolds: historically, our lab members have always seeded on one side first, waited 5-10 minutes then flipped the scaffold and

seed another amount on the other side. What we discovered in our experiments is that this technique showed several problems: it is often hard to flip the scaffold over, and while flipping the scaffold, a certain amount of cell suspension will be lost. Therefore, in this study, we discovered that seeding on one side only was more efficient, reproducible and effective. This may be different with different cell types/size/characteristics.

In this study and in the following chapters, we thus decided to seed on a dry scaffold, on one side only, at about 50M cells/mL, with the optimal volume found empirically.
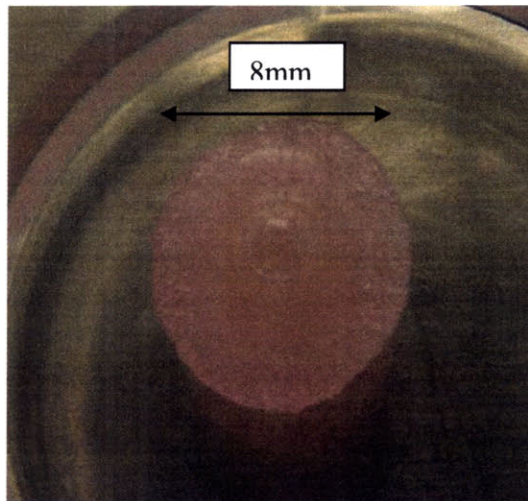
Fig.4.1. Scaffold that was just seeded with cells. Cells suspension is still visible on the top of the scaffold.

e. Analysis of Scaffold properties

i. Scanning electron microscopy

Scanning electron microscopy (SEM) is often used to image the microscopic structure of scaffolds and to evaluate the pore size, pore aspect ratio, wall size and shape. For example, when designing a scaffold for nerve regeneration pore geometry and interconnection is very important. These factors may be less critical when designing a scaffold for regeneration of the IVD.

Below are scanning electron micrographs of the scaffolds that we used in this chapter and the following (animal model).

Acc.V Spot Magn   Det WD ⊢——————————⊣ 100 μm
15.0 kV 3.0 200x   GSE 15.3 0.9 Torr
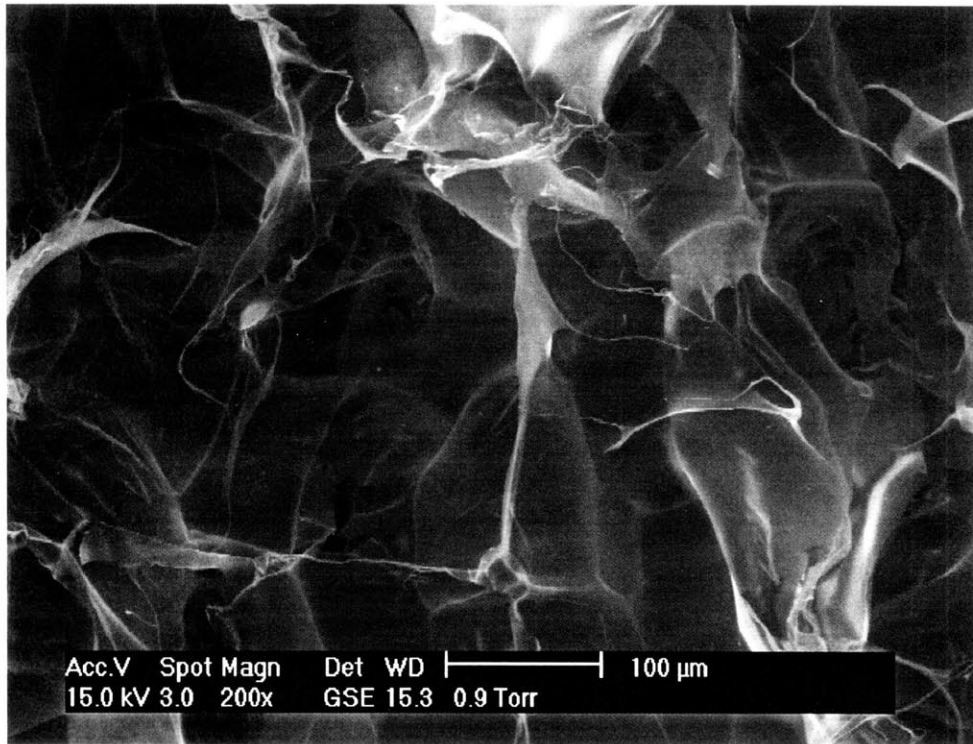
Fig.4.2a view of the large pore side of the scaffold (exposed to the air while freeze drying)

Acc.V Spot Magn   Det WD ⊢——————————⊣ 100 μm
15.0 kV 3.0 200x   GSE 16.9 0.9 Torr
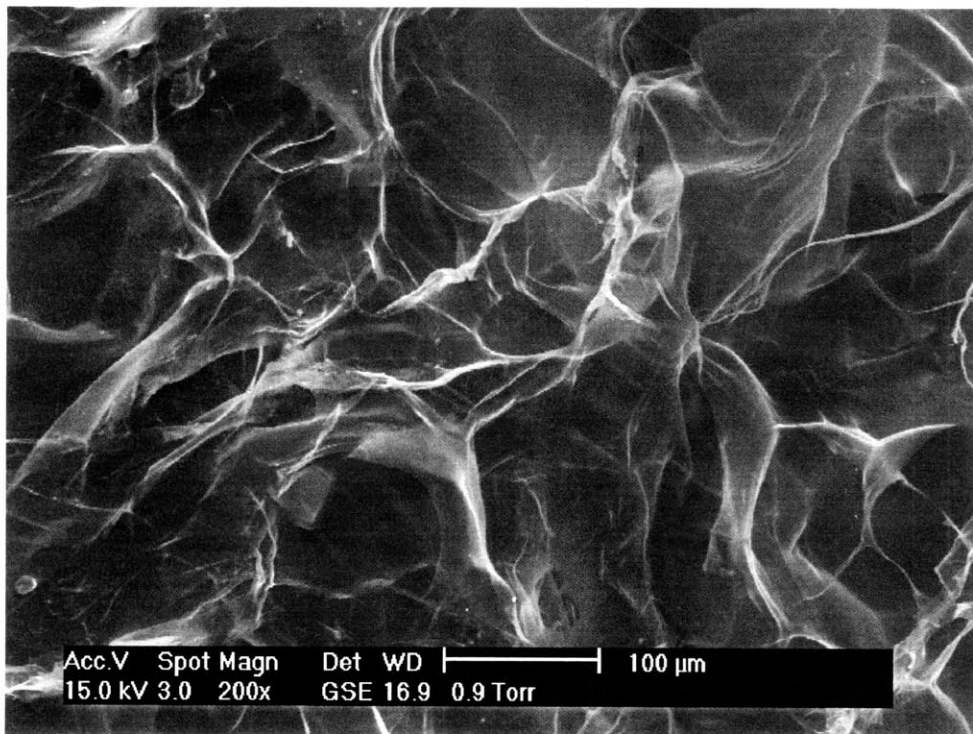
Fig.4.2b view of the small pore side of the scaffold (exposed to the mold while freeze drying)

ii. Dimensions

Scaffold dimensions was assessed using a small ruler or template.

iii. Mechanical Properties

Mechanical testing of these types of collagen scaffolds was done ([18-20]) in a few studies. Although mechanical properties are critical in certain applications (bone regeneration for example), they may be less important when considering small defects of the annulus and were not explored in this research.

iv. Biochemical

Biochemical composition of the scaffold was determined by enzyme-linked immunosorbent assay (ELISA), and DNA and GAG assays, the goal being to confirm the collagen purity and the amount of GAG after incorporation inside the scaffold.

v. Pore characterization

Pore size is very important for cell proliferation, attachment and migration. This factor was not studied in this research as a satisfactory pore size was reached (around 150µm). It was shown in previous studies ([21]) that variations of pore size by a factor of two did not yield significantly different scaffolds.

vi. Swelling Ratio

Denatured collagen behaves like rubbers ([22]) and is obtained by heating the matrices above 80C. Swelling ratio can represent a measure of the cross-link density as was shown by ([23]). Swelling ratio was not measured in this study.

f. Experimental goal

The main goal of this chapter was to find the scaffold make-up and handling methods that yielded the optimal construct for implantation in the shortest amount of time in the perspective of the animal trial presented in the following chapter.

Specific hypotheses for this work are:
- the presence of GAG in the construct is necessary for the maintenance of cells and for new matrix synthesis,
- the way GAG was incorporated in the scaffold is important,
- make up of the scaffold (type I vs. type II collagen) has a significant effect on the produced scaffold,
- type of cross-linking (DHT vs. EDAC) determines new matrix production,
- there is no significant difference between caprine and canine cultures.

2. Materials and Methods

Six different types of matrices were fabricated (Table 4.3) by changing the collagen type, the GAG content and the cross-linking method. Collagen matrices were fabricated by following previously published protocols for type I [24] and type II [25] collagen, with or without the addition of 10% (w/v) of chondroitin 6 sulfate. GAG was either added directly into the collagen slurry or was added during the cross-linking of the scaffolds (Tab. 4.3). The matrices were cross-linked either by dehydrothermal treatment (DHT) during 24 hours, or by EDAC in 100% ethyl alcohol for 24 hours at room temperature (protocol modified from [11]). The cross-linking treatment was used as sterilization process.

The six groups of scaffolds were the following:

#	Coll. Type	Crosslinking	GAG
1	I	EDAC	Freeze drying of a coprecipitate of collagen and chondroitin
2	I	DHT	sulfate
3	II	EDAC	None
4	II	EDAC	10% (w/v) chondroitin sulfate added during crosslinking
5	II	DHT	Freeze drying of a coprecipitate of collagen and 10% (w/v) chrondroitin sulfate
6	II	DHT	None

Tab. 4.3 Description of the six groups of scaffolds used in this experiment.

Methods identical to the ones used in previous chapters were succinctly detailed here. Please refer to previous chapter if necessary.

Scaffold manufacturing and properties:
Type I scaffold was manufactured the same way as in the previous chapters. Type II collagen scaffold was prepared by first reconstituting a slurry of type II collagen by blending type II collagen sheet into 5% acetic acid. 1g of collagen sheet was used for 100mL of acetic acid solution. Pore diameter before cross-linking was around 109μm for type I and around 200μm (scaffold will be analyzed in more detail in the future) for type II scaffolds. In type II collagen scaffolds, 10% of GAG was added either directly in the slurry before freeze drying, or by solubilizing GAG in water and adding it to the EDAC cross-linking solution, as was done by [26].

Cross-linking
DHT Crosslink:
- Matrices were cross-linked following the same protocol as in the previous chapter

EDC Crosslink:
- Matrices were cross-linked directly in a solution of 6mmol EDAC + 1.2mmol NHS per gram of collagen (each scaffold is assumed to be about 5mg), by mixing 0.138g EDAC + 0.033g NHS in 40mL of 100% ethanol (at -20C). Matrices were then cross-linked for 30 minutes at room temperature. Matrices were then washed twice in PBS and kept overnight at 4°C in PBS before seeding.

Cells sourcing and handling:
AF cells were enzymatically isolated from 3 adult canine IVDs and expanded separately through 2 passages in 20% FBS medium. Two million P2 cells were seeded by a pipetting technique onto dry disks 8mm in diameter and 3mm thick using around 40uL of medium + cells. Cells were seeded on one side of the scaffold only. Serum free culture medium was composed of DMEM/F12 medium (1% ITS+1, 1% BSA, 1% antibiotic, 1% L-glutamine, 2% Ascorbic acid) supplemented with TGF-β1 and FGF-2 (R&D Systems, Minneapolis) each at 10ng/mL.

Construct sacrifice:
Constructs were terminated after 2 and 4 weeks, freeze-dried and digested in papain. The following parameters were then evaluated (n=3): dry weight, DNA content, newly synthesized GAG content, GAG and total protein synthesis rate retained in the scaffolds. Synthesis rates were determined by incorporation of radiolabels 3H-Proline and 35S-Sulfate for 24 hours at 10uCie/mL. DNA was measured by a Picogreen assay and GAG by DMMB assay. Paraffin sections were stained with H&E, Masson's trichrome, safranin-O and type I and II immunohistostains for histological evaluation.

Comparison between canine and caprine cells was evaluated in a separate experiment.

3. Results

There were dramatic differences in the appearances of the scaffolds after 2 weeks in culture. The lightly cross-linked DHT-treated scaffolds decreased in diameter by approximately 50% or more (Tab. 4.4). The EDAC-treated type I (Group 1) also displayed considerable contraction. At 2 weeks the number of cells in the samples reflected in the DNA content varied by nearly 2-fold (Fig. 4.5). ANOVA revealed a significant effect of scaffold type (p<0.0001) on the DNA content, which did not vary with time in all scaffolds. Among matrices of same collagen type, EDAC-treated scaffolds had significantly more cells than DHT-cross-linked constructs (both t-tests, p<0.0001). All scaffolds had approximately the same % of mass DNA except for group #5 scaffolds which had the highest value (Fig. 4.6).

Protein biosynthesis by the cells continued throughout the duration of the experiment in all scaffolds (Fig. 4.7 and 4.8). At 2 weeks, cells in type I collagen scaffolds had the highest biosynthesis rates or protein and GAG retained in the matrix. At 4 weeks, the protein synthesis rate was significantly lower in most groups (p<0.0004, unpaired t-test). GAG biosynthesis followed the same pattern as protein synthesis at 2

weeks and was significantly higher than other groups in groups 1 and 3 at 4 weeks (Fig. 4.8). Groups 2, 5 and 6, which were the cross-linked groups had a significantly lower amount of GAG biosynthesis at 4 weeks than their EDC cross-linked counterparts. Accumulated GAG in the scaffolds was the highest in group #4 scaffolds. It seemed that DHT cross-linked scaffolds could not keep the GAG that was originally contained in the scaffolds as both groups #2 and #5 had a very small amount of GAG in the scaffolds after 2 and 4 weeks.

Histology showed a very dense new matrix for scaffold group #5 (Tab. 4.4), although the scaffold had contracted to a final diameter of around 2mm. At 2 weeks, loose connective tissue was apparent in histological sections of the type II DHT/GAG scaffolds, and residual fragments of the type II material were also in evidence (Fig. 4.12). Other scaffolds did not show a significant amount of new matrix, and most of the new tissue was observed on the periphery. Immunohistochemistry for both type II and type I collagen was positive, although most of the newly synthesized matrix was type II collagen (Fig 4.11) whereas type I surrounded the cells in most slides (Fig. 4.13). GAG was mostly located around the edges of the scaffolds and surrounded the cells. Scaffolds at 4 weeks did not show any significant increase in newly synthesized matrix compared to 2-week samples.

	Formulation			Behavior	
Group	Coll.	X-Link	GAG	2-wk Diam.	2-wk new matrix, 0-+++
1	I	EDAC	+	3mm	0
2	I	DHT	+	3	+
3	II	EDAC	-	7	0
4	II	EDAC	+*	5	0
5	II	DHT	+	2	+++
6	II	DHT	-	4	+

* GAG was added during cross-linking

Tab. 4.4 Contraction and new matrix synthesis for the 6 groups of scaffolds cultured for 2 weeks in serum-free medium supplemented by growth factors.
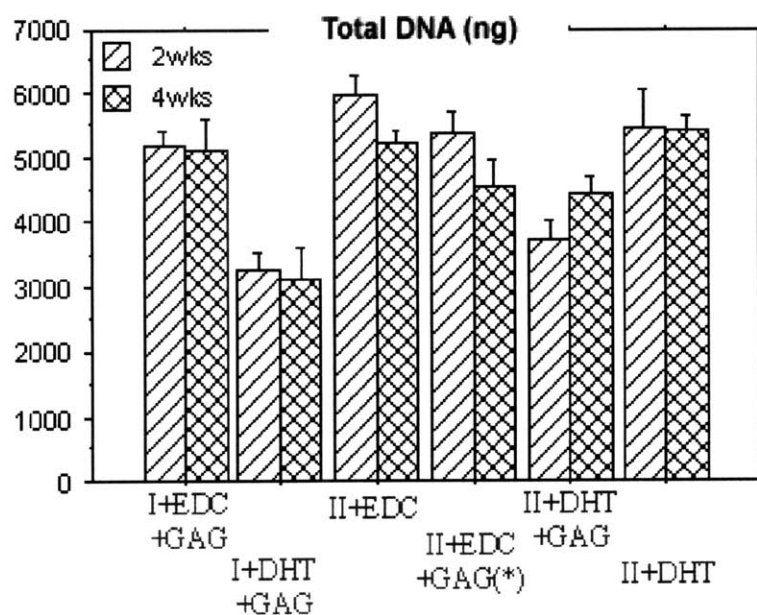
Fig. 4.5 Total DNA in the 6 different scaffolds types at 2 and 4 weeks in growth factor supplemented DMEM/F12. n=3; mean±SEM.
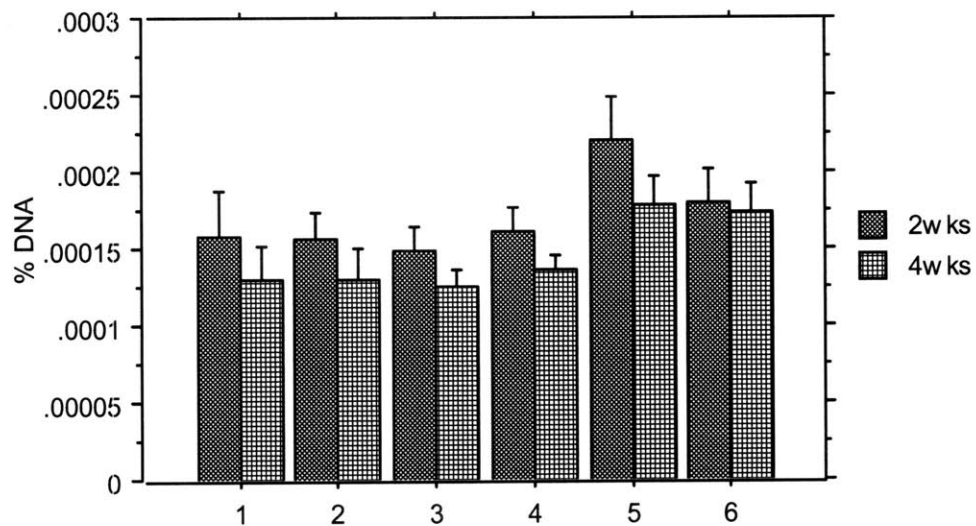
Fig. 4.6 – DNA as a percentage of mass in the 6 scaffolds group
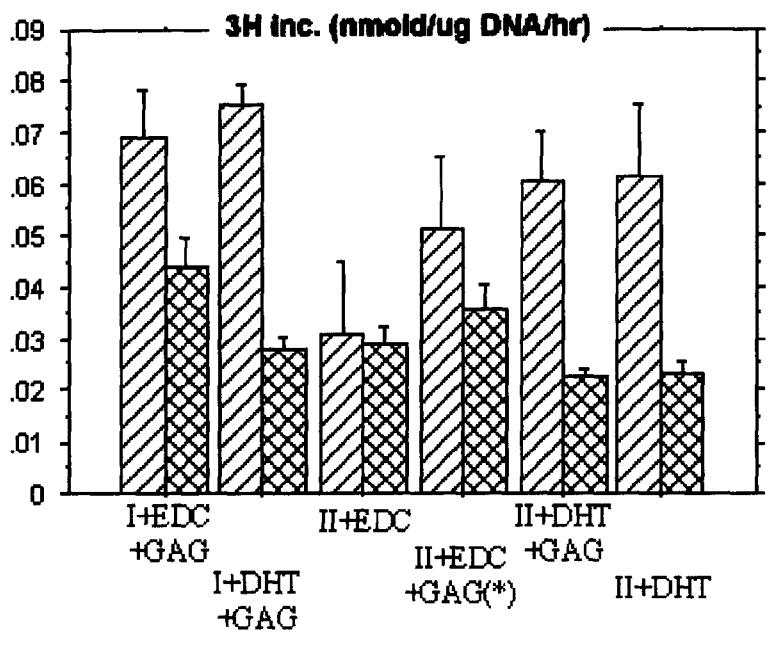
Fig. 4.7 Rate of protein biosynthesis retained in the scaffold over a 24 hour period
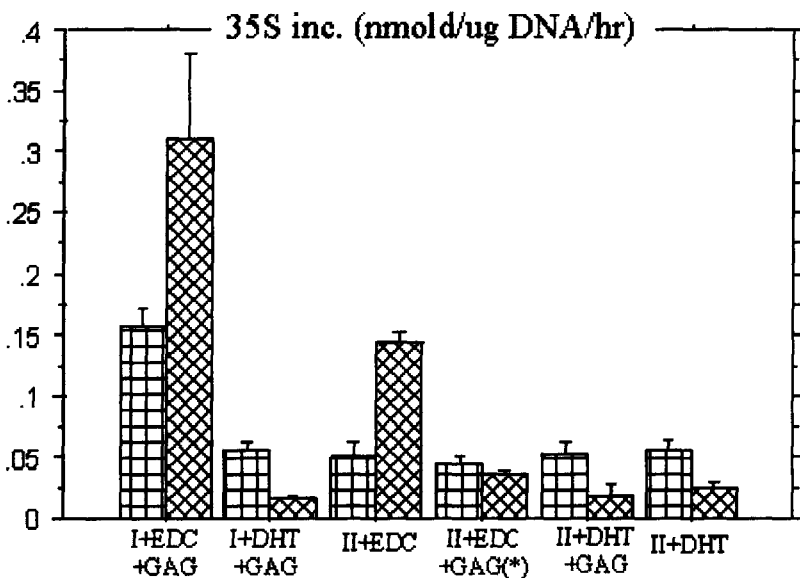
Fig. 4.8 Rate of proteoglycan synthesized and retained in the scaffold over a 24 hour period
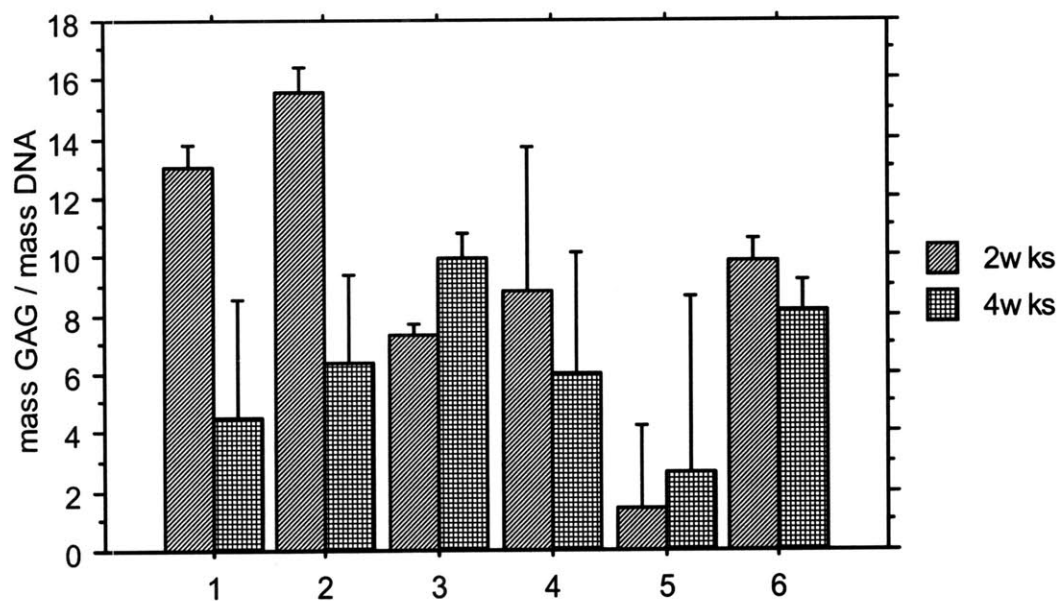
Fig. 4.9 ratio of total mass of GAG over mass of DNA for the 6 scaffolds groups.
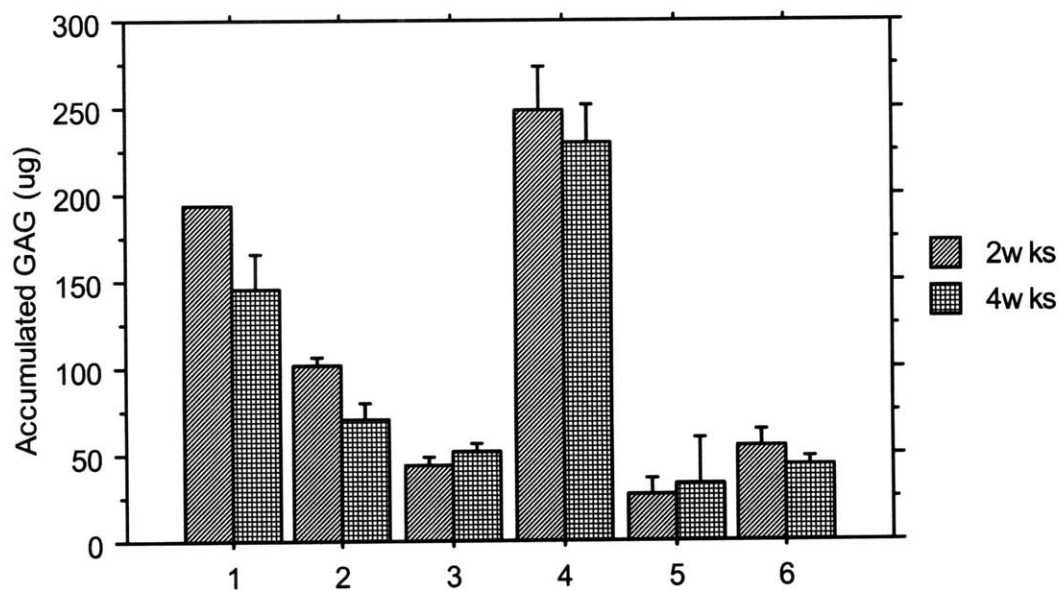
Fig. 4.10 Total accumulated GAG in the 6 scaffolds groups
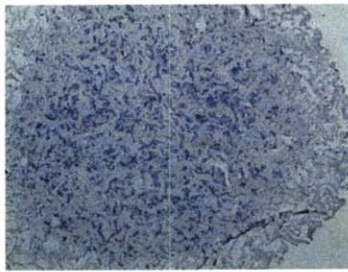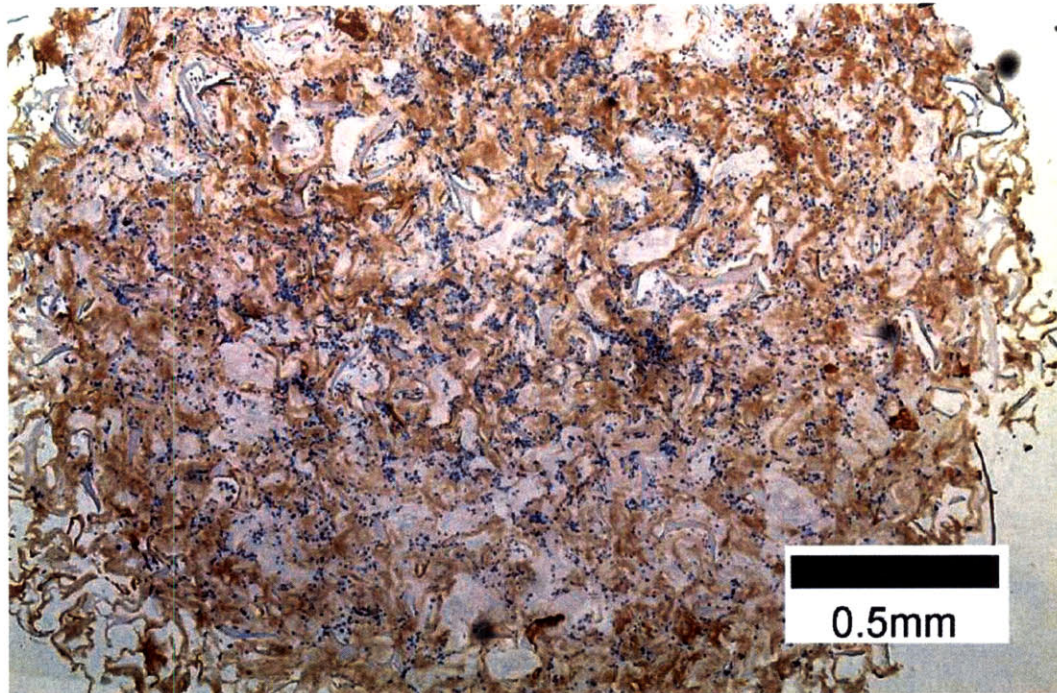
(Negative Control)

Fig. 4.11. General view of a type II – DHT scaffold at 10 days. Newly synthesized type II collagen appears uniformly spread throughout the scaffold in a loose connective tissue.
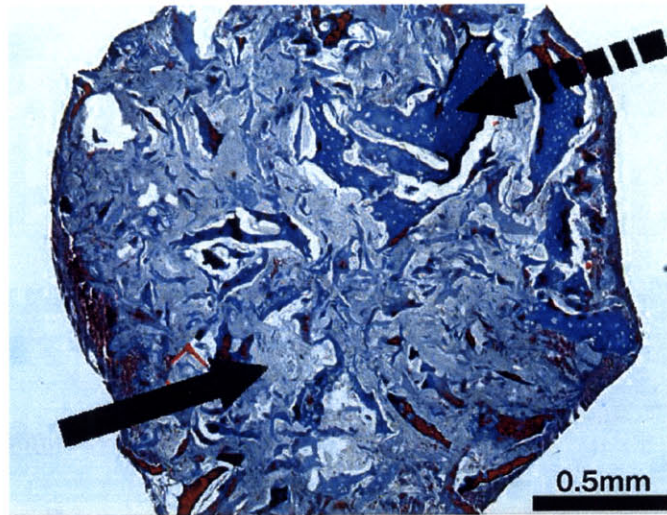
Fig. 4.12. General view of a DHT type II + 10% GAG scaffold (Masson's trichrome) at 2 weeks
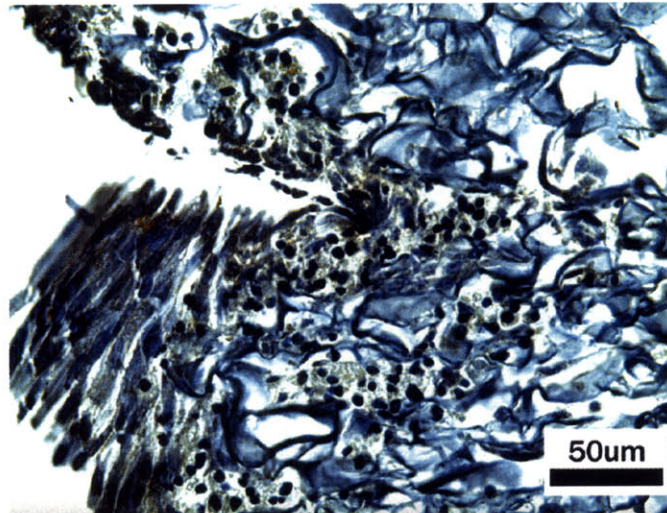
Fig. 4.13. Close up view of the edge of a #2 scaffold (type I immunostain). Cells appear surrounded by newly synthesized type I collagen.

EFFECT OF GAG

type II collagen DHT scaffold, no GAG

type II collagen DHT scaffold, 5% GAG

type II collagen DHT scaffold,10 % GAG
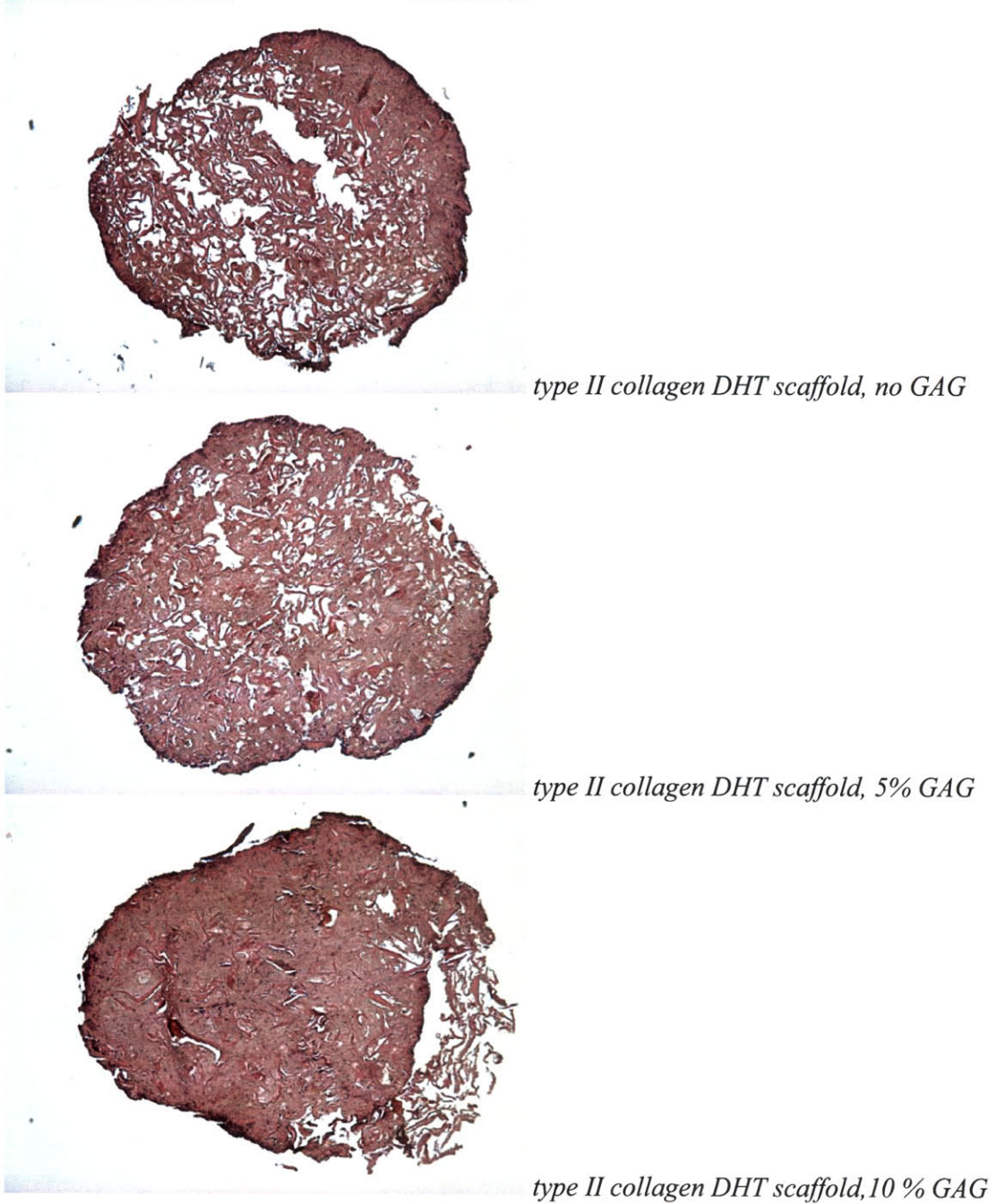
Fig. 4.14 H&E micrographs of type II collagen DHT scaffolds with no GAG, 5% GAG and 10% GAG.

Observations: the presence of GAG in the type II collagen DHT scaffolds led to a more uniform matrix with fewer tears and gaps. GAG seemed to help consolidate the matrix.

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TYPE I SCAFFOLDS (DHT)
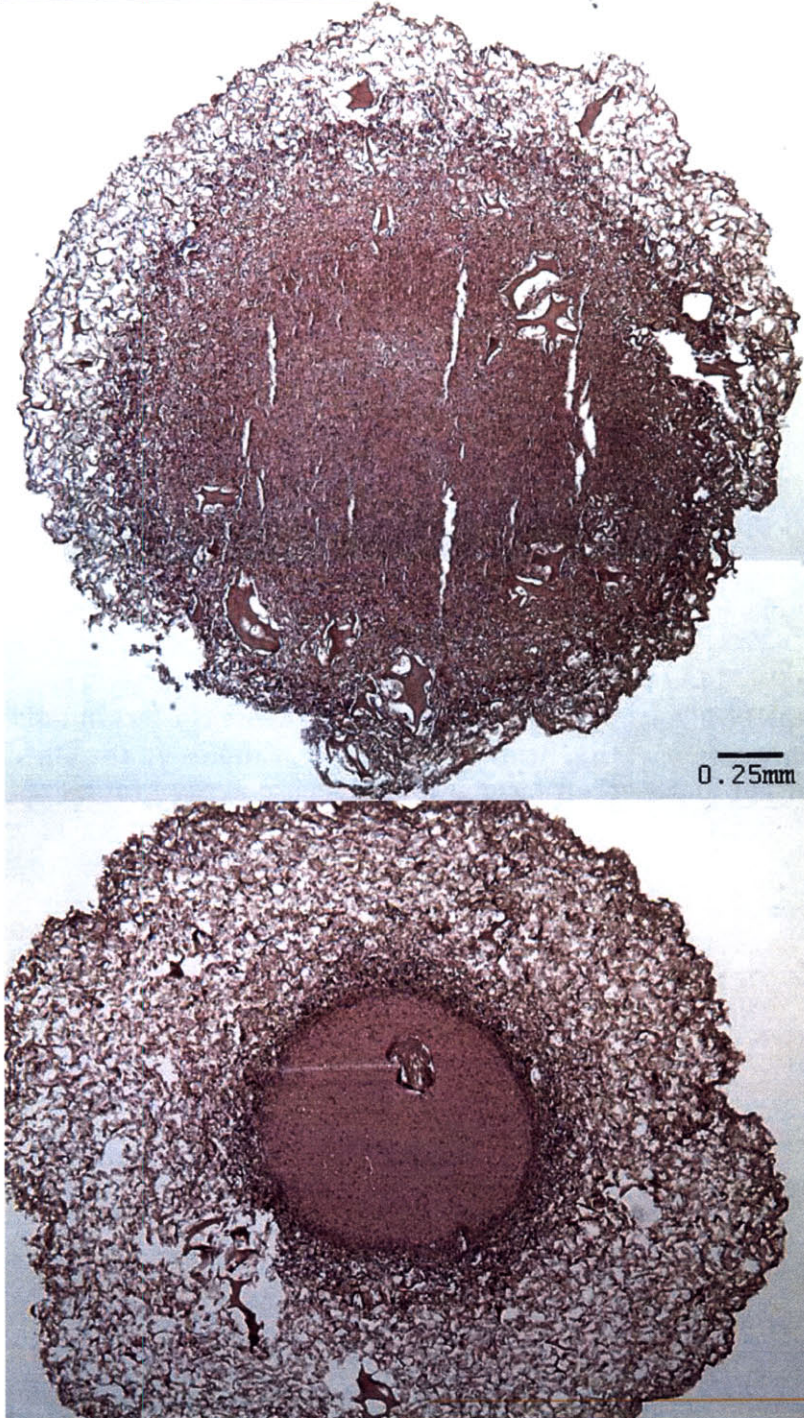
Fig. 4.15 Two constructs at 17 days - 2M cells seeded in DHT cross-linked type I collagen scaffolds

Observations: although cells seemed to proliferate well, there was little new matrix synthesized in these collagen type I DHT scaffolds. Cells uniformly spread throughout the construct.

EFFECT OF COLLAGEN TYPE

Type I (in TGF-b1)   Type II (in TGF-b1)
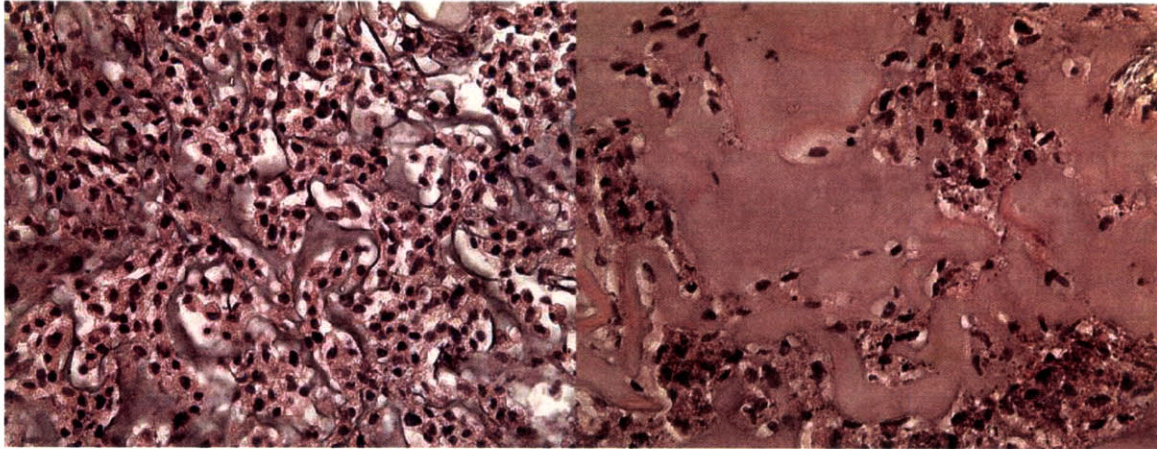
Fig. 4.16 H&E micrographs of a type I and a type II collagen DHT crosslinked scaffolds culture in serum free environment for 1 week.

Observations: cells in type I collagen scaffolds were packed in dense clusters but did not seem to synthesize much new matrix. Scaffolds in type II scaffolds on the other hand were more spread out and were in lacunae with a dense newly synthesized matrix.
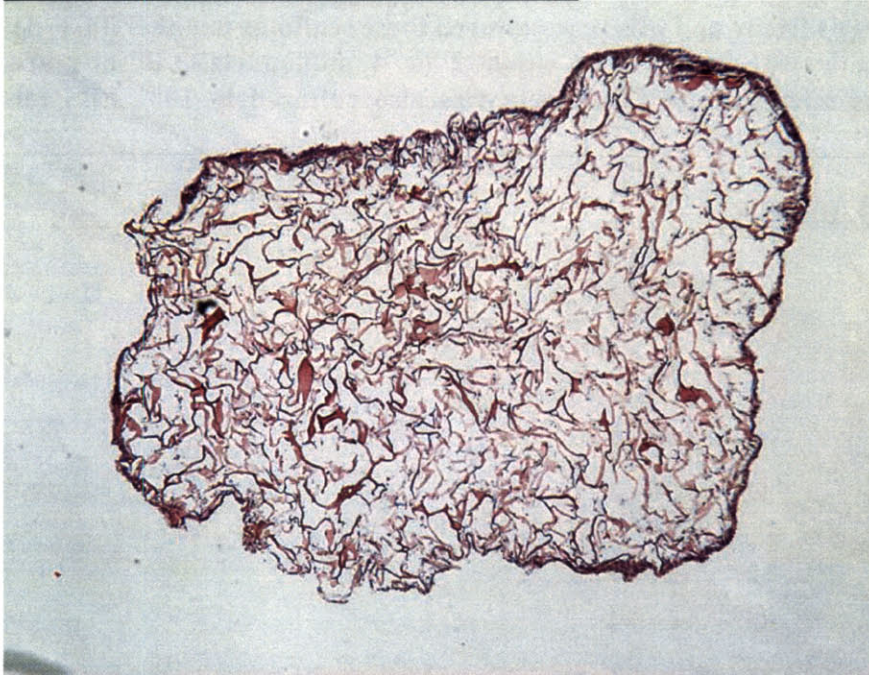
Fig. 4.17 - Four million cells seeded in EDC cross-linked type II collagen scaffold, no GAG
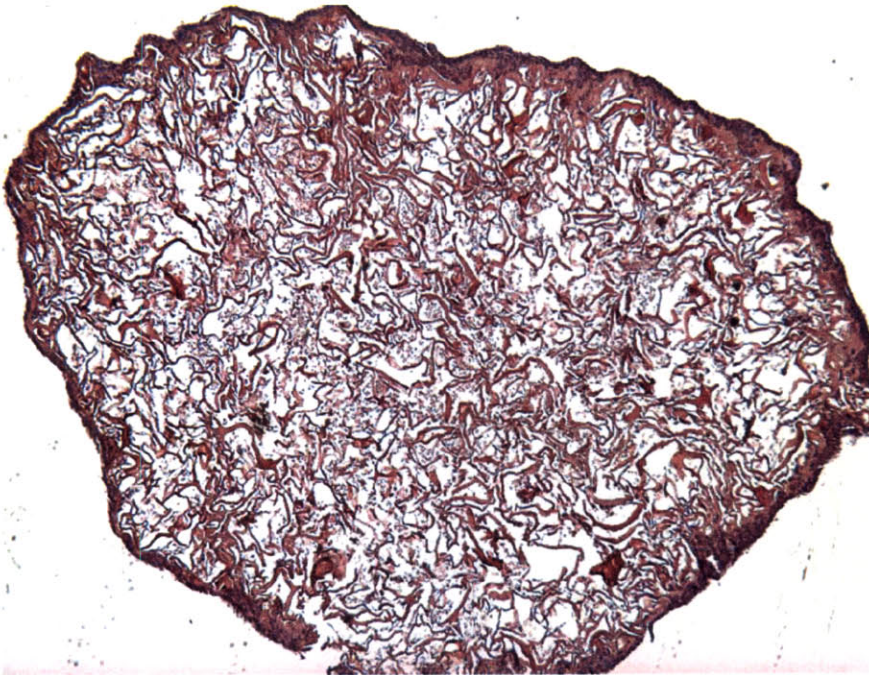
Fig. 4.18 - Four million cells seeded in EDC cross-linked type II collagen scaffold, 5% GAG

Observations: the presence of GAG in type II collagen EDC cross-linked scaffolds also seemed to yield a matrix with more newly synthesized matrix and to less contraction.

Starting from these encouraging results, we ran a similar experiment with collagen type II scaffolds in DHT only and this time cultured these scaffolds in either TGF-β1 only, FGF-2 only, or with OP-1, with either 2 or 4 million cells. Results are presented below as micrographs. One group was also cultured in 10% FBS for comparison.

EFFECT OF CELL DENSITY
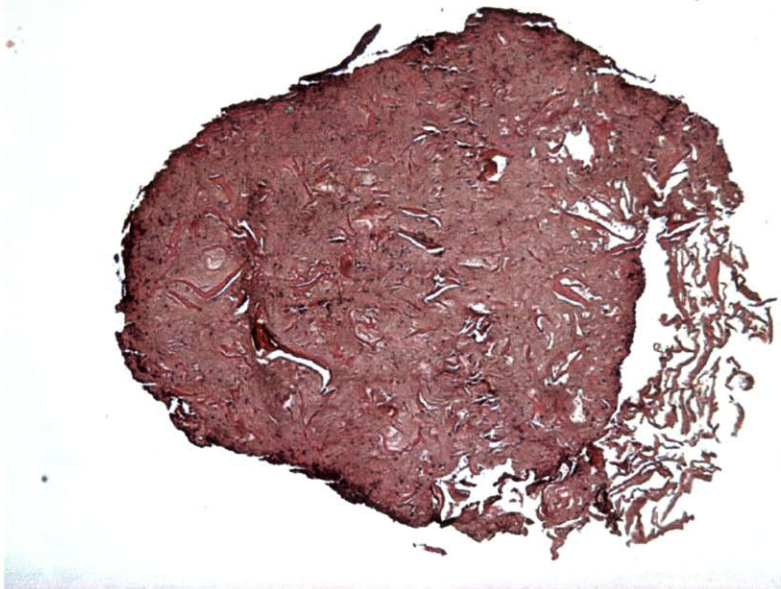
Fig. 4.19 - 2M cells seeded in DHT type II collagen scaffold, 10% GAG

Fig. 4.20 - 4M cells seeded in DHT type II collagen scaffold, 10% GAG

Observations: There did not seem to be a significant effect of cell density on the behavior of the cells in type II collagen + 10% GAG DHT scaffolds.

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EFFECT OF FBS
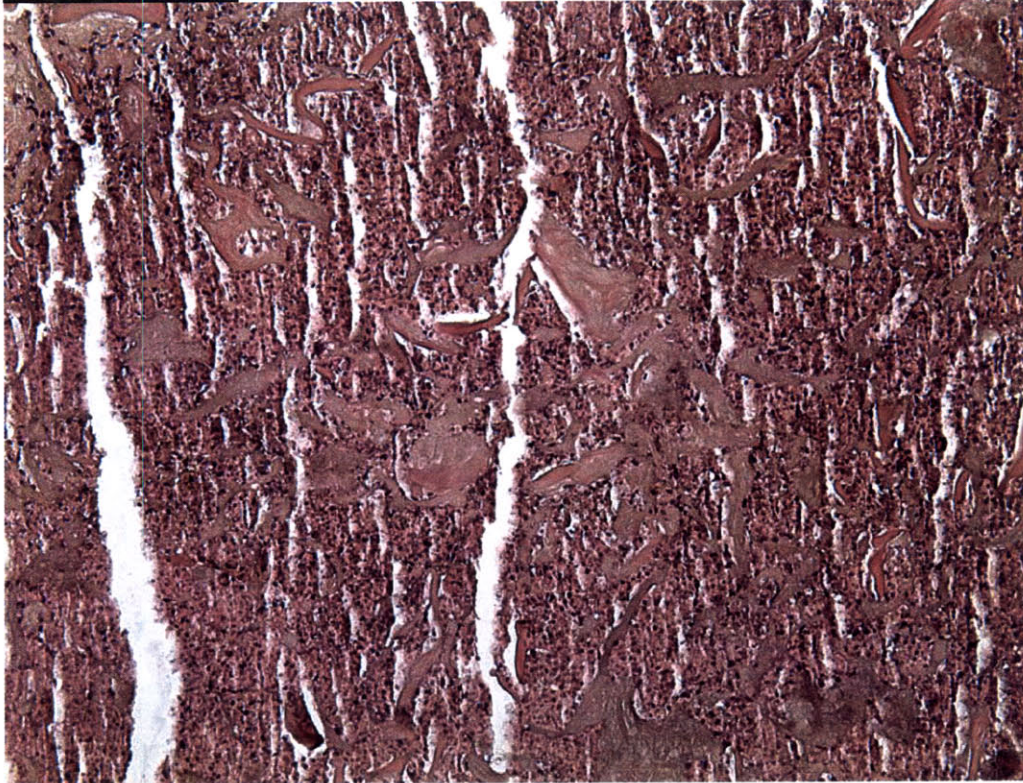
Fig. 4.21 - Detail of the center of a type II collagen + 10% GAG cultured in 10% FBS

Observations: the presence of FBS in the medium led to a strong cells aggregation in the center of the scaffold with a well defined geometric orientation.

Type II (in 10% FBS, no GF)   Type II (in TGF-b1)
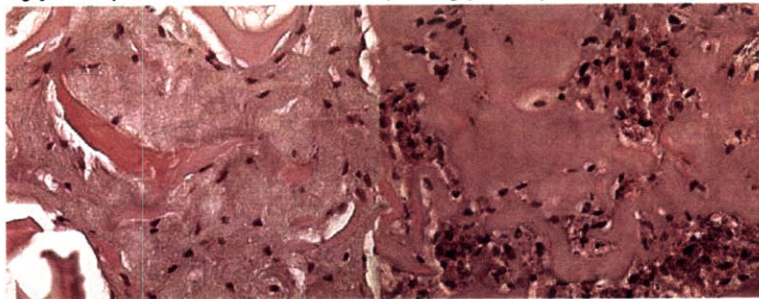
Fig. 4.22 - Comparison between type II DHT scaffolds either cultured in 10% FBS or cultures in TGF-β1 (serum-free) at 10ng/mL. More fibrochondrocytic cells are present in the TGF-β1 constructs.

Observations: it appeared very clear that TGF-β1 enables cells to retain their phenotype compared to FBS. The matrix of newly synthesized tissue also appeared denser in the TGF-β1 cultured construct.
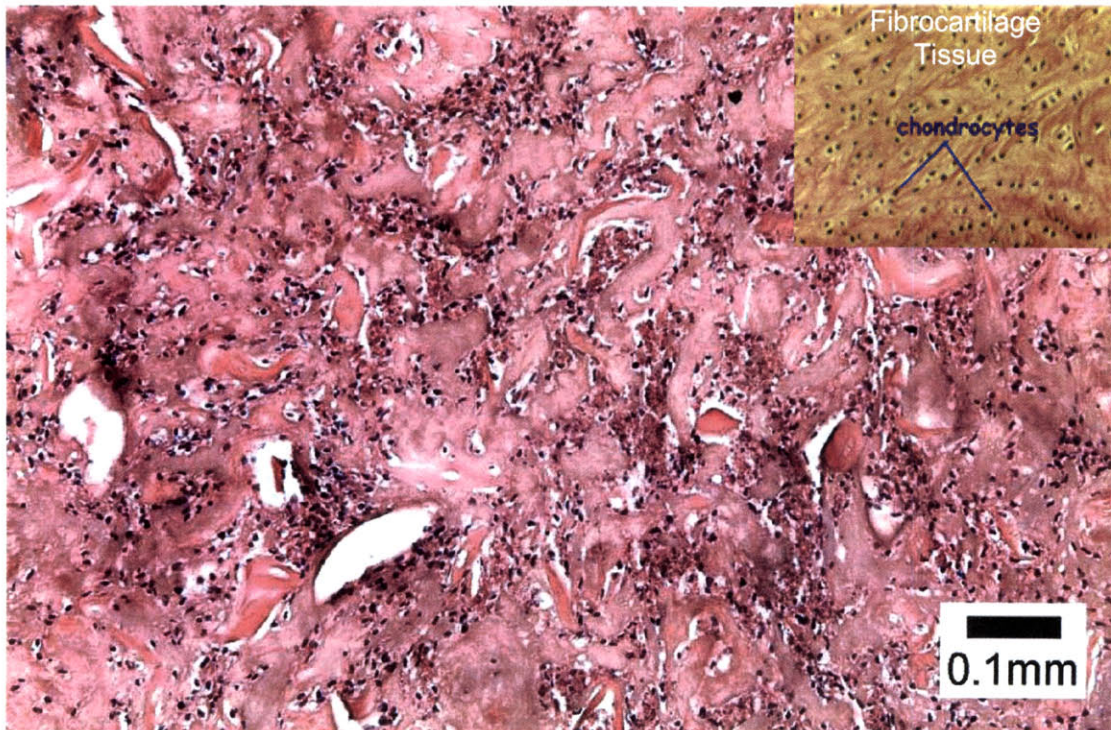
Fig. 4.23 - Close up view of a DHT type II + 10% GAG scaffold after 2 weeks of culture (H&E). Fibrochondrocytes are clearly visible.

Observations: the tissue that was synthesized closely resembles the structure of the native fibrocartilage tissue.
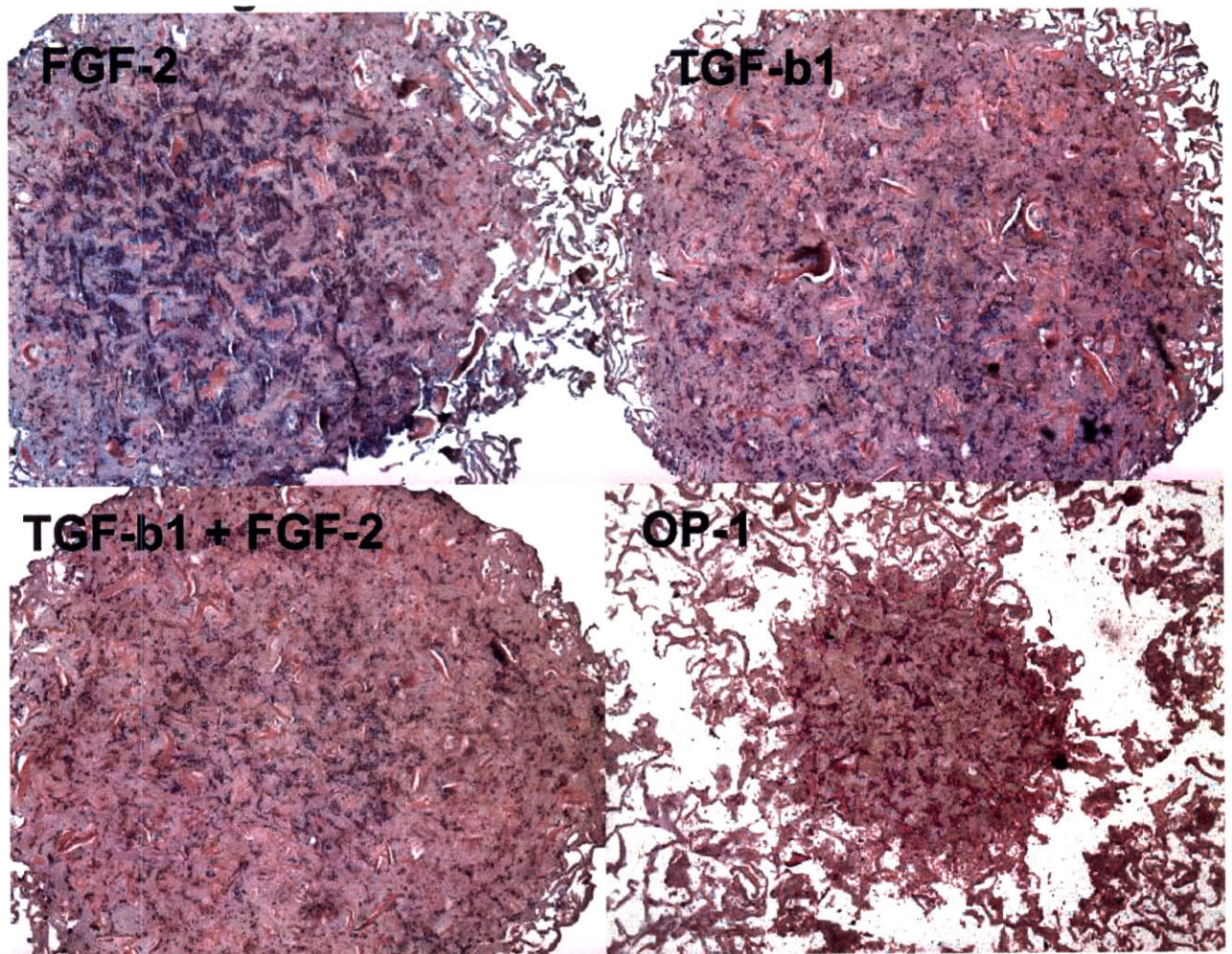
Fig. 4.24 - Comparison of constructs obtained when culturing AF goat cells in DHT crosslinked type II collagen + 10% GAG scaffolds for 1 week in either 1) FGF-2 at 10ng/mL, 2) TGFβ1 at 10ng/mL 3) a mix of TGF-β1 and FGF-2 both at 10ng/mL 4) OP-1 at 200ng/mL.

Observations: the mix of TGF-β1 and FGF-2 yielded the best result with more fibrochondrocytic cells than with TGF-β1 alone. FGF-2 alone yielded constructs with poor cell spreading and OP-1 scaffolds mostly disintegrated except a center that had a strong newly synthesized matrix.

EDAC type II scaffold

DHT type II scaffold

DHT type II + 5% GAG scaffold
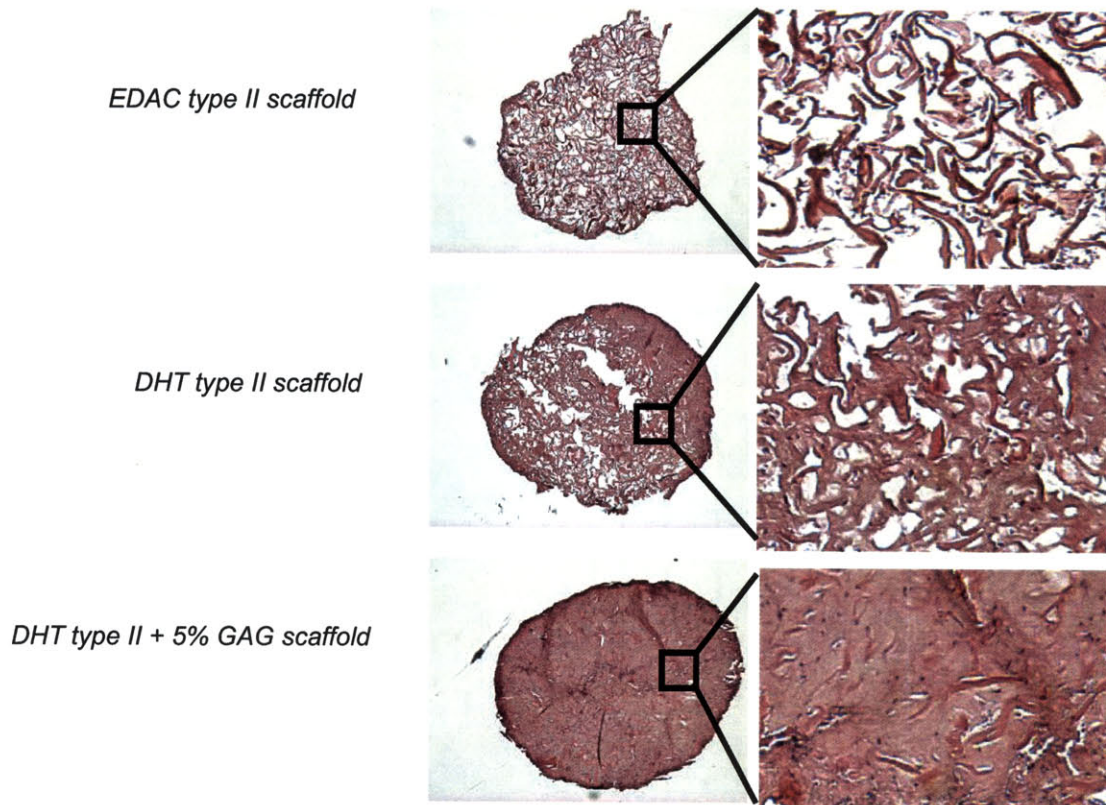
Fig. 4.25 general and close up views of 3 type II collagen scaffolds cultured for 10 days in a mix of TGF-β1 and FGF-2 : 1) EDC cross-linked 2) DHT cross-linked 3) DHT cross-linked + 5% GAG

4. Discussion

Of interest was the dramatically different behavior of AF cells in type I and II collagen scaffolds, the strong effect of the cross-linking method and the effect of the diverse growth factors.

#	Diameter	# of cells & location	New matrix
1	3mm	Few cells, mostly on edge	Very little
2	3mm	Many cells, in cluster	Very small pore
3	7mm	Few cells, uniform distrib.	Huge pore
4	5mm	**Many cells, uniform distrib.**	Very small pore
5	2mm	Very high density at some locations	**Dense new matrix**
6	4mm	Medium density, uniform distrib.	Very little

Tab. 4.26 Summary of key results (first experiment only)

Effect of:	
Collagen type	Type I: high cell number, high cell metabolism, low new matrix Type II: cells uniformly spread, dense new matrix (except in EDC crosslinked matrices)
GAG	For DHT crosslinked scaffold, GAG stabilizes new matrix
Cross-linking method	Less contraction and higher cell number in type II scaffolds (EDC scaffolds). Denser new matrix for DHT scaffolds
Seeding density	No effect between 2 and 4M cells
FBS	Strangely oriented pattern in the new matrix
Growth factors	The mix of FGF-2 + TGF-β1 seems to be the most efficient for the culture of AF cells in collagen scaffolds

Tab. 4.27 Summary of key effects

Effects of Collagen Type: DHT-treated GAG-supplemented samples type II scaffolds (#5) contained more cells and newly synthesized matrix despite a lower 2-wk biosynthetic rate compared to type I specimens (#2). The EDAC-treated type I (#1) and II (#4) scaffolds did not display such notable differences with little new matrix.

Effects of GAG: The presence of GAG did not seem to have much of an effect on the type II scaffolds that were EDAC-cross-linked (#s 3 and 4). However, for the DHT-treated type II samples, the presence of GAG appeared to result in an increase in matrix synthesis (#5 vs. #6; Table 1) despite there being fewer cells. GAG appeared to prevent tears in the scaffold resulting in a more uniform and homogenous matrix.

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Effects of Cross-Linking: For the type II matrices, the EDAC-treated scaffolds contracted less than the DHT cross-linked matrices, a result that had already been observed as EDAC-treated scaffolds are usually stiffer than DHT-treated scaffolds. For the type I scaffolds for which there was no difference in contraction (#s 1 and 2), EDAC resulted in a greater number of cells but less of a biosynthetic rate. For the type II matrices with GAG (#s 4 and 5), more newly synthesized matrix was found in the DHT-treated samples (#5). This result could indicate that scaffold contraction is necessary for the synthesis and maintenance of new matrix molecules. For the samples with no GAG (#s 3 and 6), there was no noticeable effect of cross-linking.

Effects of seeding density: Densities of 2 and 4 million cells were used but did induce significant difference.

Effect of FBS: Scaffolds cultured in FBS had a strange geometrically oriented pattern in the middle of the scaffolds, making these constructs probably unsuitable for implantation.

Effect of growth factors: What was observed in the FBS scaffolds did not appear in the growth factor supplemented serum free medium. The mix of TGF-β1 and FGF-2 both at 10ng/mL yielded the best result with more fibrochondrocytic cells than with TGF-β1 alone. This mix should be the medium composition of choice for future experiments. FGF-2 alone yielded constructs with poor cell spreading and OP-1 scaffolds mostly disintegrated except in the center where they showed a dense newly synthesized matrix.

We can now look at the hypotheses we made at the beginning of this chapter:
- the presence of GAG in the construct is necessary for the maintenance of cells and for new matrix synthesis: **rejected**. The presence of GAG is beneficial for the type II collagen DHT scaffolds but is not necessary for the maintenance of the cells and of the new matrix.
- the way GAG was incorporated in the scaffold is important: **rejected**. We cannot say for sure it was important. However, incorporating the GAG during cross-linking resulted in a higher level of GAG in the scaffolds after 4 weeks.
- make up of the scaffold (type I vs. type II collagen) has a significant effect on the produced scaffold: **accepted**. Cells cultured in type II collagen scaffold DHT crosslinked kept their phenotype and synthesized a fibrocartilagenous matrix.
- type of cross-linking (DHT vs. EDAC) determines new matrix production: **accepted**. There was very little new matrix production in EDC cross-linked scaffold despite the higher number of cells.
- there is no significant difference between caprine and canine cultures: **accepted**. Within the scope of the experiments we made, there was no histologic difference between constructs made using goat cells and constructs made using dog cells.

The results of this study are encouraging. It is the first report of the maintenance of the fibrochondrytic phenotype of the AF cells in a collagen scaffold. Although collagen orientation is not yet similar to the tissue lamella orientation, we suggest that the construct will remodel in vivo. Compared to previous chapters, the construct that was made was much more uniform, contracted by just 50% (second experiment) after 1 week with no important additional contraction between 1 and 2 weeks. The construct appeared ready within 10 days of culture for implantation (with GAG content at around 60% of the native GAG content of the annulus fibrosus (100ug/mg of dry tissue in the goat AF tissue), making this process a suitable one for medical application. One limitation is that type I collagen was not much synthesized in the scaffold and that type II collagen was preponderant. This is different from what is observed in the native tissue where type I collagen is more abundant than type II in the annulus, although this is often considered to be the case because type I is synthesized during degeneration.

Conclusion: The findings of this study underscore the design challenge in selecting the collagen type, GAG content, and cross-linking method for the formulation of the most suitable AF cell-seeded scaffold for implantation. A selected type II scaffolds (*e.g.* #5) could be commended on the basis of the newly synthesized matrix, but had the disadvantage of the contracted dimensions. Type I constructs could be chosen on the basis of the number of cells (#1) or the high biosynthetic activity at 2 weeks (#2). Future *in vivo* evaluation of these scaffolds will help to inform the selection algorithm.

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CHAPTER 5 – ANIMAL MODEL: IMPLANT OF AN AUTOLOGUS CELL-SEEDED COLLAGEN-GAG SCAFFOLD IN A GOAT MODEL

1. Background on animal models

a. Main challenges

In the previous chapters we designed, manufactured and optimized the culture of collagen constructs seeded with AF cells. Now, picking the optimal animal model to evaluate the regenerative potential of this collagen-GAG scaffold appears as a real challenge. Tradeoffs will be necessary as there does not exist a perfectly similar animal with intervertebral discs having the same geometric, mechanical, degenerative and nutritional characteristics as the human disc.

i. scaffold

Naturally, we picked the scaffold developed and improved in chapter 4 for this study. This type II collagen, DHT cross-linked scaffold, seeded with P1 cells and cultured for 10 days in serum free medium supplemented with TGF-β1 and FGF-2 seems to be a very good candidate for implantation (see below).

ii. time of implantation

An important parameter is the time of implantation of the scaffold. Although certain studies report immediate implantation of the cell-seeded scaffold at the site of surgery with no prior *in vitro* culture, others first start by cultivating the scaffold *in vitro* for a certain amount of time before surgical implantation. This discrepancy can lead to dramatically different results as shown in this study ([1]) on a dog model for articular cartilage regeneration.

Time of implantation is challenging in several ways:
- problems of implanting too early: this could lead to partial or no regeneration as the implanted tissue would have difficulty to be integrated in the existing tissue
- problems of implanting too late: the mature tissue would be less likely to be reshaped in vivo to form a living tissue, increasing the chance of rejection and the quality of synthesized tissue.

Finding the right time for implantation is difficult and there is currently no adequate technique for universally deciding when an engineering tissue is ready for implantation. Modeling such time would require a perfect understanding of the tissue synthesis,

degradation and integration with the *in situ* tissue. This knowledge is unfortunately far from perfection.

In this study, we decided to culture the cells for a period of 10 days in the collagen-GAG scaffold, exactly as done in chapter 4 and to implant the seeded scaffold into a standardized defect that was just created in the animal. This scaffold is suitable for implantation for several reasons:
- cells are uniformly spread in the scaffold
- new matrix has been synthesized and is quite dense after 10 days with a few original pores left.
- construct contains 50 to 60% of GAG per mg of tissue of the native AF goat tissue.
- cells have maintained their phenotype as confirmed by the presence of type I and type II collagen and the fibrochondrocytic appearance of the cells.
- scaffold can easily be handled by a surgeon, it will not break or collapse when held by forceps.
- due to its low cross-linking density, the scaffold has already contracted by 50% and will not contract much subsequently as shown in prior studies that extended up to 4 weeks
- time in culture was suitable for a medical application.

iii. animal model

Most animal models have tried to regenerate the disc and to identify the induction factors of its degeneration. Kroeber for example, in a rabbit model, induced degeneration with a series of controlled axial mechanical loads ([2]). Other studies involving rabbit animal models [3] use a needle to puncture the annulus as a standard defect to induce degeneration. Kim *et al.* compared the effect of three disc injury models on a rabbit IVD ([4]). These injury models which included a) the injection of an apoptotic agent, b) nucleus aspiration and c) annulus puncture. Lipson and Muir ([5]), demonstrated that puncture of the disc led to annular disorganization, osteophyte formation and fibrous replacement of the nucleus in a rabbit model. Sobajima *et al.* studied in detail the reproducibility of the degeneration created by these types of punctures using MRI, X-Ray and histology techniques ([6]). Other animal models include bovine models, with the work of Demers *et al* ([7]) where biochemical content of bovine and human discs were compared. Meisel *et al* used a dog model to evaluate the potential of disc chondrocytes transplantation ([8]).

Following annular injury, the region experiences loss of proteoglycans and water content which eventually leads to fibrous replacement of the annulus and increased collagen production, mostly type I, II and IV ([9]). Other matrix metalloproteinases molecules are also produced in larger quantity ([10]). Granulation tissue is present in the region of injury [11, 12] and is innervated and vascular in regions low in proteoglycans. The presence of growth factors such as FGF and TGF-α as well as pro-inflammatory cytokines such as TNF-α is also to be noted ([13, 14]). This granulation tissue is thought

to play an important role in discogenic pain ([15, 16]) and in healing, as they allow the passage of macrophages into the degenerated tissue ([17]). Indeed, studies have shown that the peripheral annulus heals by first closing itself with this granulation tissue ([18]), then by synthesizing new nuclear tissue. This regeneration process allows partial recovery of the original pressure contained in the nucleus. However, large defects are much slower to heal and defects may become worse with movements, injury and other mechanical stimulations on the disc.

In a review by Lotz ([19]), animal models are classified either as "induced" or "spontaneous", involving animals that will display disc degeneration spontaneously or after genetic modification. Induced disc degeneration can be done either mechanically (compression/instability) or structurally (defect). This is this latter approach that was favored in this study.

Considering our ultimate goal, preparing an autologous AF cell-seeded scaffold for the repair of a standardized defect close to the human, it appears necessary to look in detail at two parameters: disc size and cells type.

Disc size matters because diffusion of nutrients through the disc is mainly done by diffusion, and a small disc will be less affected by the avascularity of the disc than a large disc. For example, rats which have biochemical properties close to the humans ([20]) will be less affected by nutrients diffusion than humans.

Cells type matters because many small animal discs contain notochordal cells ([21]) which is often thought to prevent disc degeneration by increasing nucleus pressure and resisting disc pressure. Adult human disc do not contain any notochordal cells.

The disc size of a goat is very similar to a human. Besides, goat discs do not seem to contain notochordal cells (European Cells and Materials Vol. 10 Suppl. 3, 2005 (page 59)) and experience degenerative changes in response to spinal injuries. These reasons make the goat model an attractive one.

The goat model was also picked over other models because of:
- availability
- price
- ease of procurement of tissue for isolation of cells
- annular defects similar in size to those occurring in humans can be made
- high tolerance to surgery

GOAT
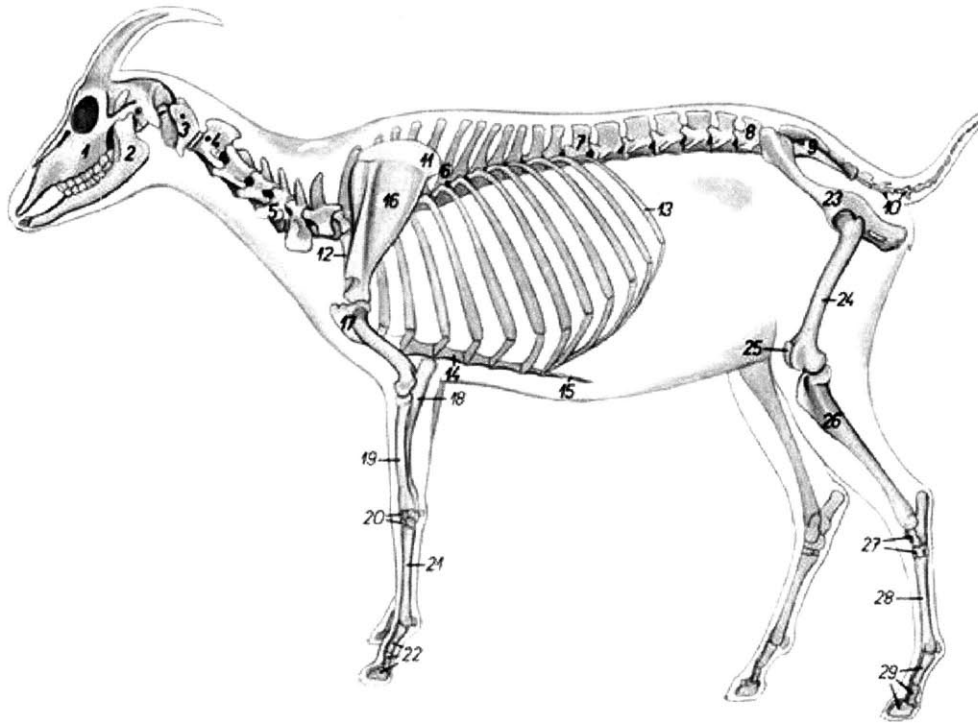
Fig. 5.1. Goat skeleton

iv. Reparative evaluation

Although the most important assessment is pain, it is impossible to measure pain in animal models. Traditionally, assessment of the repair was done by either MRI, X-Ray, histomorphometric analyses, immunohistochemistry, gross morphology, and mechanical testings. These techniques were used in all the references mentioned in this chapter. These techniques appear useful in the sense that, contrary to many other tissues, the morphology of the disc is related to its functionality and to the pain.

In this study, we evaluated the quality of the regeneration vs. the controls by gross morphology, X-Ray and histomorphometric analyses.

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b. Experimental goal

The goal of this experiment was to evaluate the potential response of an autologous cell-seeded collagen type II-GAG scaffold, cultured *in vitro* for 10 days before implantation in a standardized defect. Several (six) groups, each made of six animals were planned in order to evaluate:
- The effects of a sham surgery where the disc and ligaments are exposed,
- The natural response after 2 and 4 months to a standardized defect created in the annulus fibrosus,
- The regenerative response after 2 and 4 months to a standardized defect in which was implanted an unseeded collagen-GAG scaffold,
- The regenerative response after 2 months to a standardized defect in which was implanted an autologous cell-seeded collagen-GAG scaffold as prepared in chapter 4.

Specific hypotheses for this work are:
- surgical implantation is feasible,
- sham operation may lead to future degeneration of the disc,
- repair of the damaged disc is enhanced by the implantation of a cell-seeded collagen-GAG scaffold,
- A scaffold alone induces an inferior regeneration than a cell-seeded scaffold,
- Scaffold will integrate into the surrounding tissue.

2. Materials and Methods

a. Experimental Design

Eighteen goats will be used for the implantation procedures to be performed in this study (see table). Each goat will be operated twice, once on the left side and at the higher L2-L3 level and two months later at the lower right side of L4-L5. The goats will be divided into 6 groups:
- Sham operation (group 1, n=6) where the disc and the ligament will be exposed only. Animal will be sacrificed after 4 months.
- Annulotomy (Group 2, n=6), where a standard defect will be created in the annulus of the disc. Animal will be sacrificed after 2 months.
- Annulotomy with implantation of an unseeded collagen-glycosaminoglycan (CG) matrix (Group 3, n=6). Animal will be sacrificed after 4 months
- Same as group 3, but animal will be sacrificed after 2 months (Group 4, n=6)
- Same as group 2, but animal will be sacrificed after 4 months (Group 5, n=6)
- Implantation of an autologous annulus fibrosus (AF) cell-seeded CG matrix (Group 6, n=6)
- Group 0 consisting in unexposed, untouched disc will also be used to observe intact discs.

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The sham procedure is required because of reports that surgical intervention on a disc, even without an annulotomy, can cause degenerative processes to result.

These six groups are useful to evaluate the self reparative response of the disc to a standardized defect, either alone or in the presence of an unseeded or seeded construct.

Implantation of an unseeded matrix will help us determine how cells contribute to the integration of the implant and overall healing.

Detailed description of the six groups: the first group (Group 1) will receive a sham operation in which the disc at L2-L3 will be exposed but not incised. Two months later, this group will have a discectomy performed at L5-L6 (group 2 or A2). For the six animals in Group or AS4, discectomy will be performed at L2-L3 initially and at L5-L6 2 months later (group or AS2), with unseeded collagen matrices implanted at both levels. In the six animals in Group 5 or A4, disc tissue will be resected from L2-L3 in the first surgical procedure to yield cells for seeding matrices to be implanted in a defect produced at L5-L6 in the same animal 2 months later (group 6 or ACS2). The discectomy site at L2-L3 will remain empty.

The animals will have their neurovascular status closely followed in the postoperative period. All animals will be sacrificed 4 months after the index procedure.

Group #	Level	Sacrifice date	Surgery	Animal #
0	varied	N/A	untouched	various
1	L2-L3	4 months	Sham	516-519-515-521-83-19
2-"A2"	L4-L5	2 months	Annulotomy	516-519-515-521-83-19
3-"AS4"	L2-L3	4 months	Annulotomy+Unseeded Scaffold	52-43-71-63-56-73
4-"AS2"	L4-L5	2 months	Annulotomy+Unseeded Scaffold	52-43-71-63-56-73
5-"A4"	L2-L3	4 months	Annulotomy+Annulus Harvest	137-101-78-58-98-77
6-"ACS2"	L4-L5	2 months	Annulotomy+Seeded Scaffold	137-101-78-58-98-77

Tab. 5.2. Table showing the different groups

b. Cells isolation and expansion

Tissue samples obtained from the annulotomy of each goat were first rinsed in PBS, then minced, then placed in a collagenase type I and type II solution composed of DMEM/F12 (25mL) and of 0.1g/50mL of collagenase type I and 0.1g/50mL of collagenase type II. The digesting solution was placed in a small tissue culture flask which was then spun on a rotator to allow the tissue to be digested overnight. The next morning, cells were filtered through a 40um filter and spun down. They were then washed in medium twice. Cell number was counted and depending on the number of cells, cells were then plated in one or two 25cm^2 tissue culture flask until confluence in DMEM/F12 with 20% FBS. Confluence was reached in about 10 days. At this point, cells were passaged and frozen in liquid nitrogen for future use. They were thawed 20

days before the scheduled date for the next surgery, grown until confluence (reached after around 7 days) then seeded in scaffolds and cultured subsequently for 10 days, at which point the cell-seeded scaffold was implanted.
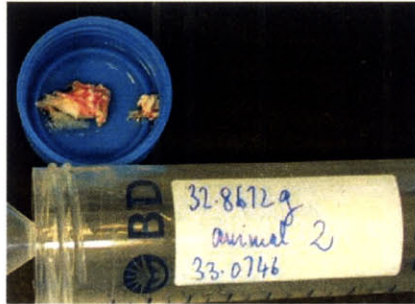

Fig. 5.3. Biopsy used to isolate the cells. This biopsy corresponds to the tissue of the standardized defect. In this case, 203mg of tissue was extracted.

c. Scaffold Manufacturing

Same technique was used as in chapter 3. The sheet obtained was extremely porous (large pore) on the side that was exposed to the air whereas the other side has smaller pores.
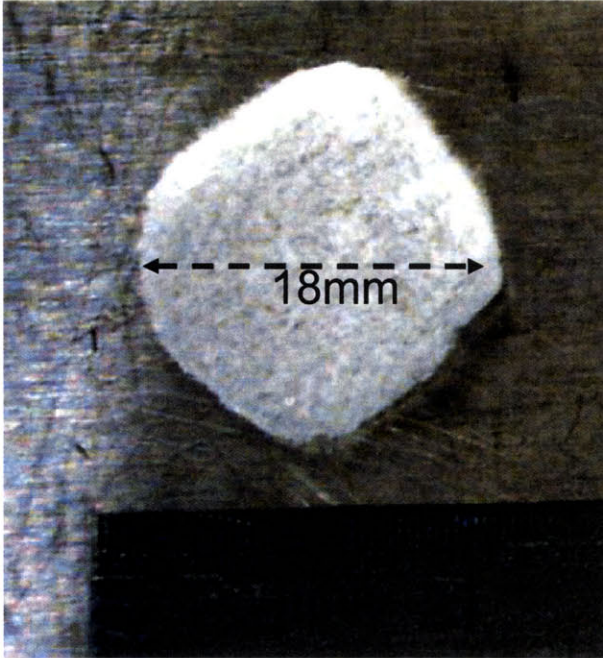
Fig. 5.4. 2cm diameter cut scaffold, ready for seeding.

d. Cells Seeding

The technique used for cell seeding was similar to previous chapters. However, for these large scaffolds, we ran several pilot experiments to see whether this would be better to seed in one time a large amount of medium or if we had to seed in several times. It seems the optimal volume to seed was around 240uL. We seeded on the large pore side of the scaffold. We decided after several trials to seed 3 times 80uL at 3 different locations on the scaffold (see figure below). After 10 minutes of seeding the scaffold looked uniformly seeded. 8 to 16 million cells were seeded in the scaffold according to cells availability per animal.
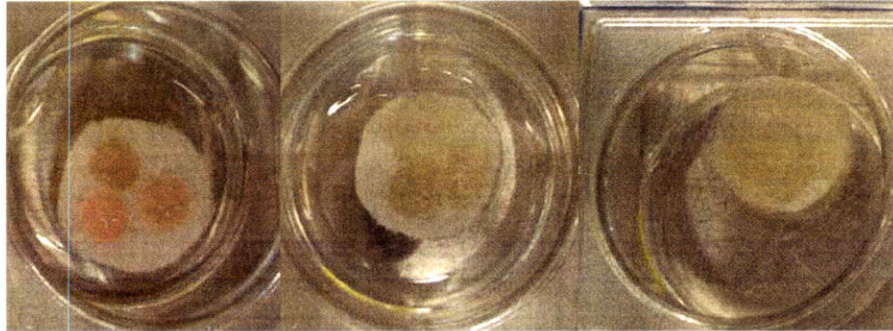
Fig. 5.5. Seeding of 8-16million cells in 3 times 80uL in these 2cm diameter scaffolds (left). After 5 minutes (center) cells suspension has started to spread. After 10 minutes (right) the scaffold looks uniformly seeded.

e. Scaffold Culture

After a couple of hours, we added 0.5mL of medium (containing 10% FBS) and after overnight incubation, we added 2.5 mL additional medium. At the next medium change, serum-free medium supplemented with the mix of TGF-β1 and FGF-2 was used. Scaffolds were cultured for a total of 10 to 12 days before the day of implantation. For each animal, 2 scaffolds were prepared and the morphologically better looking scaffold was used for implantation, the other one was used for histology.

Fig. 5.6. - 4 two-cm diameter type II collagen-GAG scaffolds in serum free medium supplemented with a mix of TGF-β1 and FGF-2.

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f. Surgical Implantation

The day of the surgery, the tissue culture plate was wrapped with parafilm in order to prevent non sterile air to contaminate the samples. After the site of implantation was exposed and confirmed on an x-ray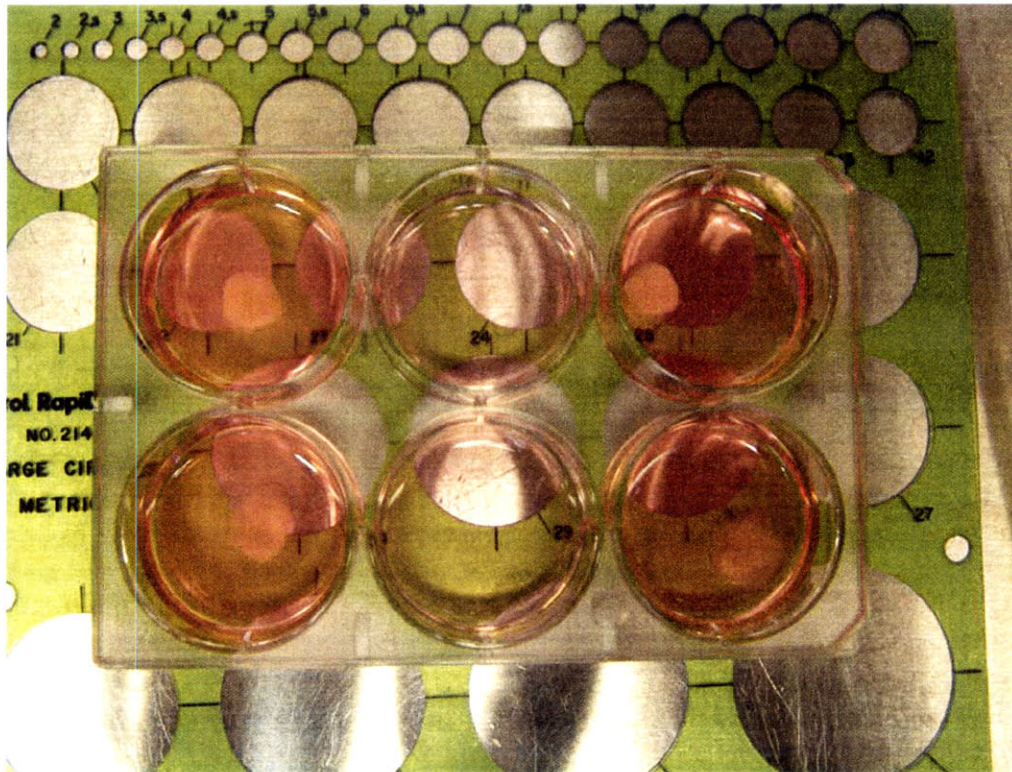, the constructs were transported from the tissue culture room into the operating room. The standardized defect was then made in the annulus. Consequently, one of the two scaffolds was removed from the medium and trimmed to a rectangle of around 9mm x 5mm in size (see figures). The scaffold was then placed at the site of the defect. In certain cases where the defect thickness was larger, another scaffold was used. The defect was covered by an unseeded scaffold. The remaining seeded scaffold was placed in formalin for histology.
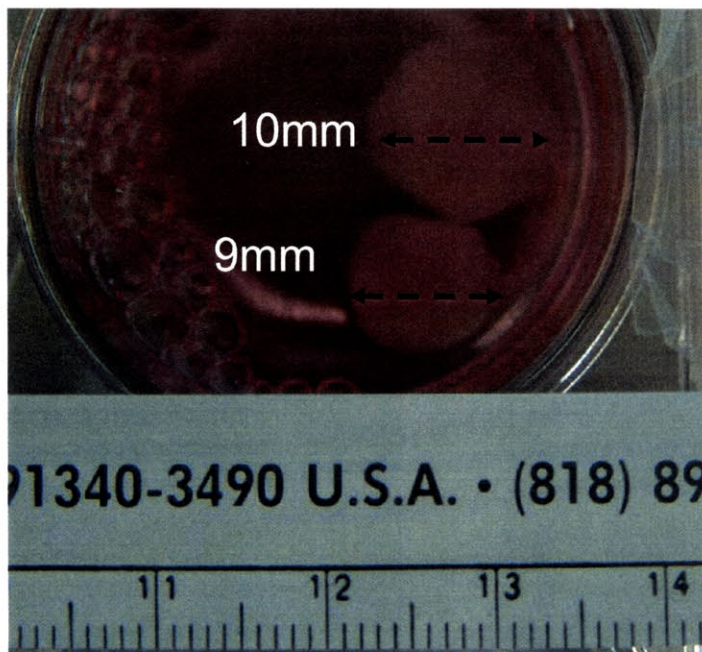

Fig. 5.7. the two cell-seeded scaffolds just before implantation. Parafilm covers the tissue culture plate to prevent infection.
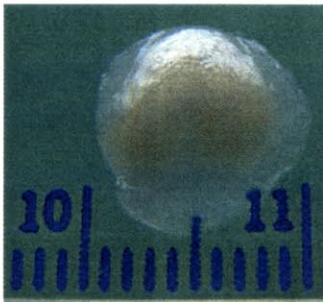

Fig. 5.8. cell-seeded scaffold after it has been removed from the medium. One can clearly outline the location of the cells and of the new matrix and the edges that do not contain many cells.
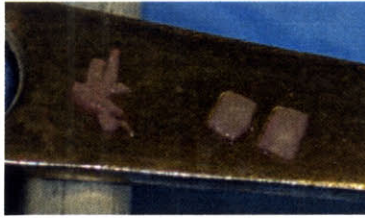
Fig. 5.9. the two cell-seeded scaffolds ready for implantation after they have been trimmed with a scalpel. On the left are visible trimmed edges.

Details of the surgical procedure (Discectomy and Implantation) (from Animal research protocol)

The left flank and abdomen were draped in the standard fashion. The procedure was be carried out through a retroperitoneal exposure. The exposure was limited to either the L2-L3 or L5-L6 levels. By performing the procedures below the level of L1, injury to the spinal cord was limited. By selecting surgical sites far apart in the lumbar spine, any detrimental effect an injured disc can have on an adjacent level was minimized. The sham procedure consisted of exposure of the disc and the anterior longitudinal ligament. Discectomies and harvest procedures were performed via approximately a 3 mm x 5 mm rectangular annulotomy in the midline of the disc space. The depth of the annulotomy was approximately 1 cm. The second procedure was performed from the opposite side. As in all anterior exposures, care was taken to avoid penetration of the peritoneum and to identify the segmental vessels and ureter. Soft tissue insertion into the endplates was preserved, as is typically done clinically. The same types of defects were produced for the harvest and implant sites so that a matrix implanted into the donor site for 4 months could be used for comparison with the 2-month implanted matrix, albeit their location in discs was at different levels. Unseeded and cell-seeded collagen implants were placed in the site of defect and covered by an unseeded matrix sheet to prevent tissue penetration in the disc. Implantation of the matrix had been practiced in several canines during necropsy to test this procedure and in a pilot study of 3 dogs. The appropriate analgesics were administered to the animals as needed.

Analgesic, sedative, or anesthetic used, plus dose, route, and duration. Sedatives: Atropine, 0.05mg/kg, subcu. Ketamine, 20 mg/kg, IM. Xylazine, 0.1 mg/kg, IM. [Optional: Thiopental, 10mg/kg. IV.] Anesthesia: Isoflurane with O2, 1-2% endothracheal intubation Analgesic: Banamine 1-2mg/kg IM BID for the first 48 hrs. after surgery and then as needed.Antibiotic: Cefazolin, 35mg/kg, intravenous
Sedatives: Atropine, 0.05mg/kg, subcu. Ketamine, 20 mg /kg, IM. Xylazine, 0.1 mg/kg, IM. [Optional: Thiopental, 10mg/kg. IV.] Analgesic: Banamine 1-2mg/kg IM BID for the first 48 hrs. after surgery and then as needed.
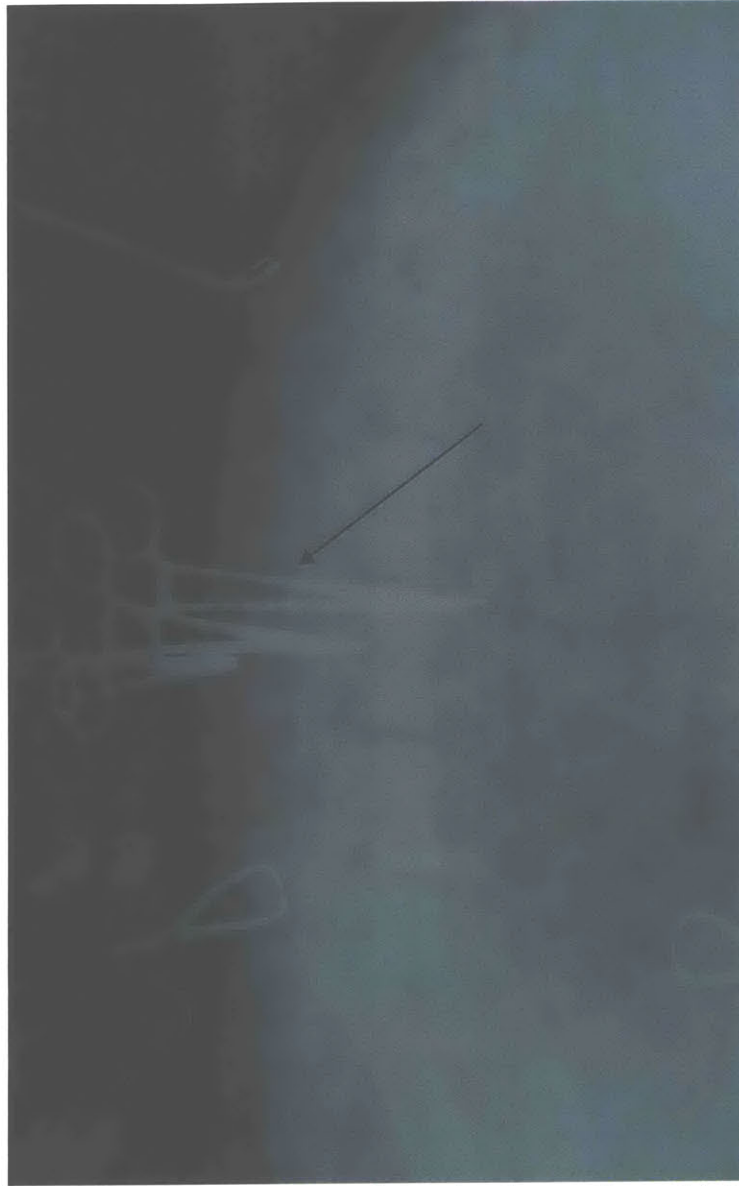
Fig. 5.10. X-ray taken during surgery and showing the needle used to localize and confirm the L4-L5 IVD.
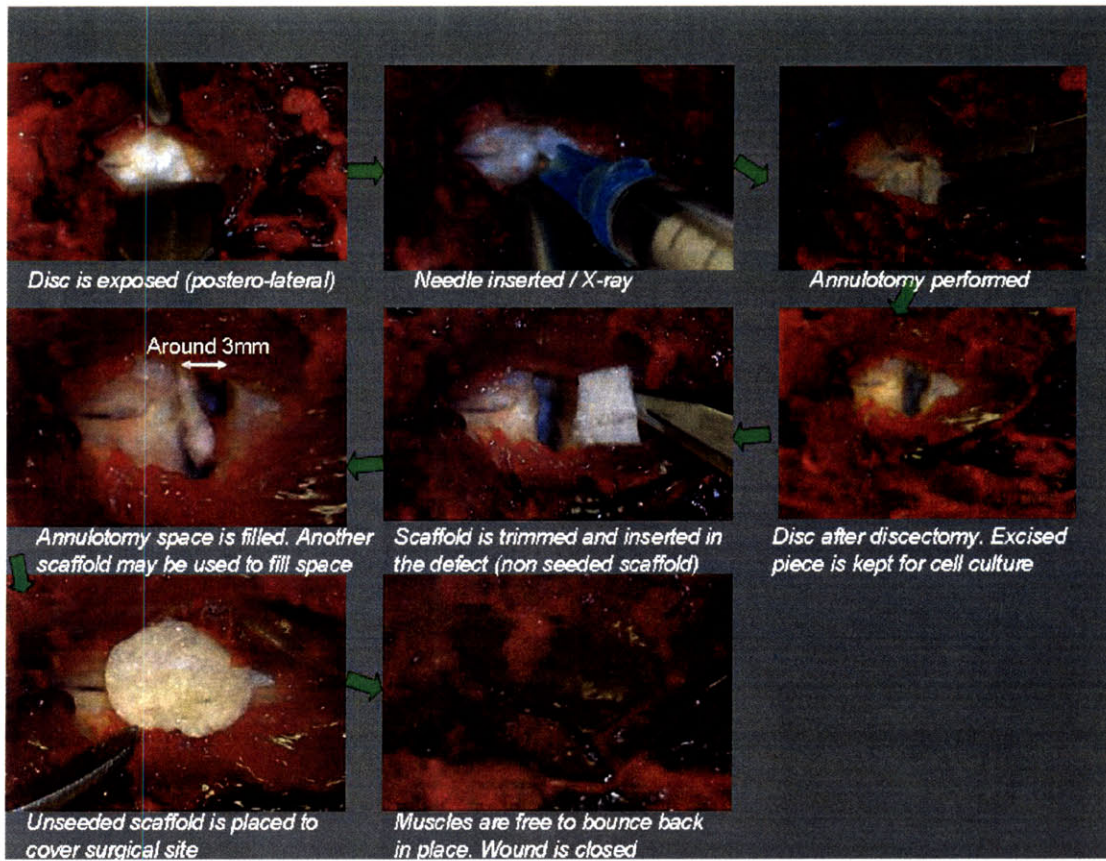
Fig. 5.11. Detail of the surgical procedure
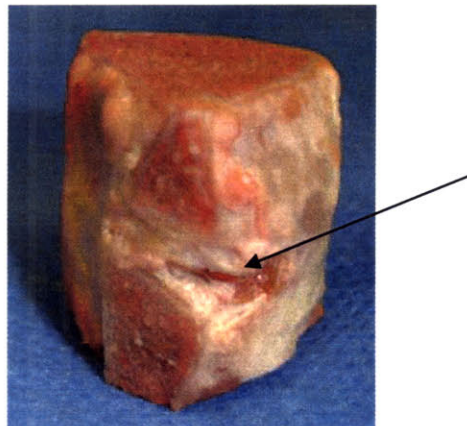
Fig. 5.12. Detail of a spinal segment with the standardized defect pointed by the arrow

g. Animal Care

Animals were taken care in the animal facility of the VA hospital.

h. Animal Sacrifice and extraction of site of implant

On the day of sacrifice, animals were videotaped and photographed to show that they had no neurological or physical problem. They were first anesthetized and then received a lethal injection of pentobarbital through the carotidal artery. Death was immediate. Following death, animal was shaved and spinectomy was performed. Immediate x-ray (dorsal and lateral) was taken to locate and mark the sites of surgery (L2-3, L4-5).
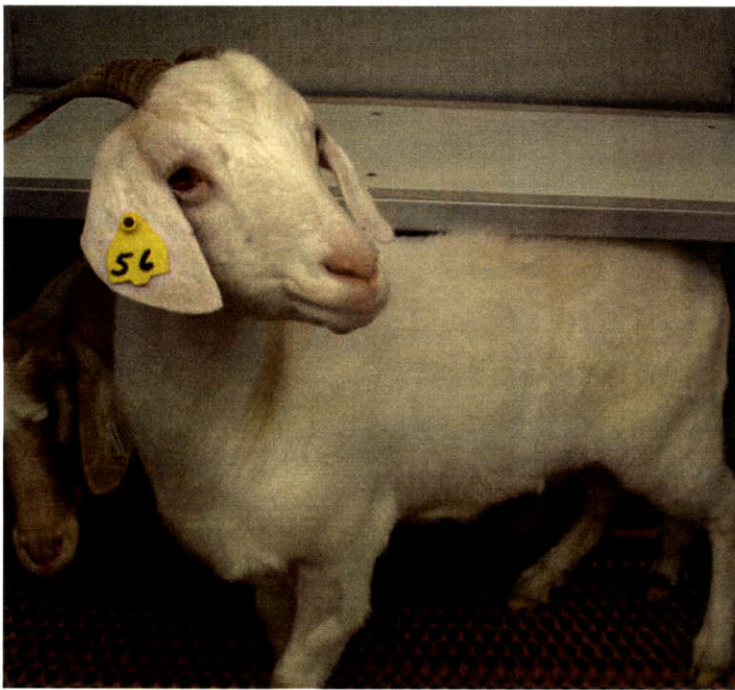
Fig. 5.13. Goat #56 photographed before sacrifice.

i. X-ray and disc height

X-ray was taken to measure disc height. The spine was placed directly onto the film so that direct measurement of disc height could be possible. Possible ossification of the disc was also looked for when analyzing the x-ray.

j. Fixation of site of implant and decalcification

Spine ligaments and muscles were then removed while preserving soft tissue around the operated site. Using a vertical band saw, disc segments were cut through the adjacent

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vertebrae as close to the disc as possible. These whole segments were then placed in formalin for 3 weeks. After this period of time, the segments were cut closer to the disc and were placed in a decalcification solution (10% EDTA) for periods of at least 2 months. Decalcification solution was replaced twice a week for a fresh one and bone was trimmed as much as possible at each solution change with a razor blade.

k. Histological processing

Once segments were soft enough for processing, they were cut in a standardized fashion (see figure) in order to obtain sagittal and transverse cuts of the disc at the level of the defect. These cuts were then photographed for gross morphology, before being processed in a tissue processor (Hypercenter XP, Shandon) and embedded in paraffin. Sections, 7 µm in thickness, were then cut. The microtomed sections were stained with hematoxylin and eosin, safranin-O (for proteoglycan), and Masson's trichrome (for collagen). Slides were cover-slipped and sealed with cytoseal 60 cell mounting medium.

l. Histomorphometric evaluation

Micrographs of the sagittal sections were taken and the regeneration was evaluated by measuring the areas of the different kinds of tissues contained within cross-sections through the standardized defect. As the defect could not be identified precisely, a standard rectangular region of interest that covers the defect was outlined as follow: the verteberal bones on the annular side, 1/3 of the width of the disc into the disc, 1/10 of the thickness of the disc on the superior and inferior sides of the disc. This ensured that the region of the defect was covered in all the different scaffolds.
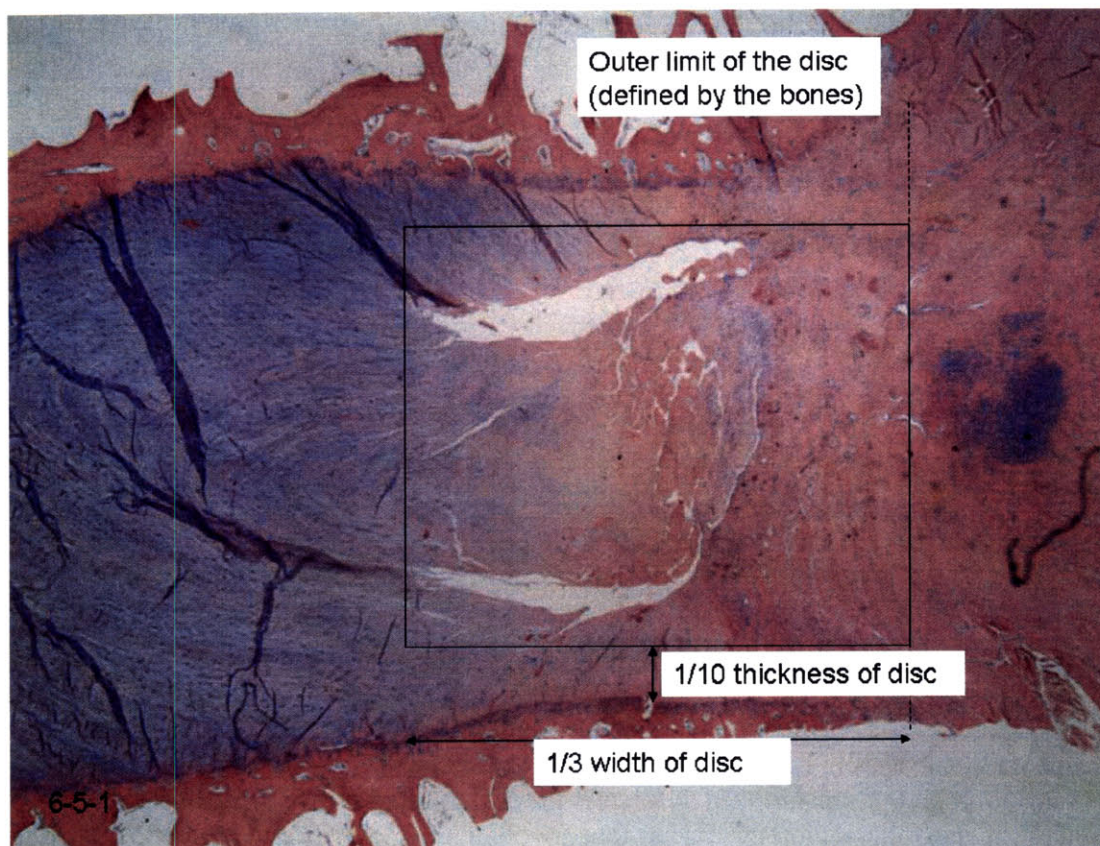
Fig. 5.14. H&E stain of a Sagittal cut of an animal of group 6. Standard region of interest was shown as well as its boundaries.

Once this region of interest (ROI) was outlined, the different types of tissues present in the ROI were identified and their relative areas were compared to the total area of the ROI using the imageJ software. Several types of tissues could be identified:

- Fibrous Tissue
- Fibrocartilage
- Cyst
- Nucleus Tissue
- Intact annulus lamella
- Blood vessels
- Red blood cells infiltrate

The original scaffold was not visible.
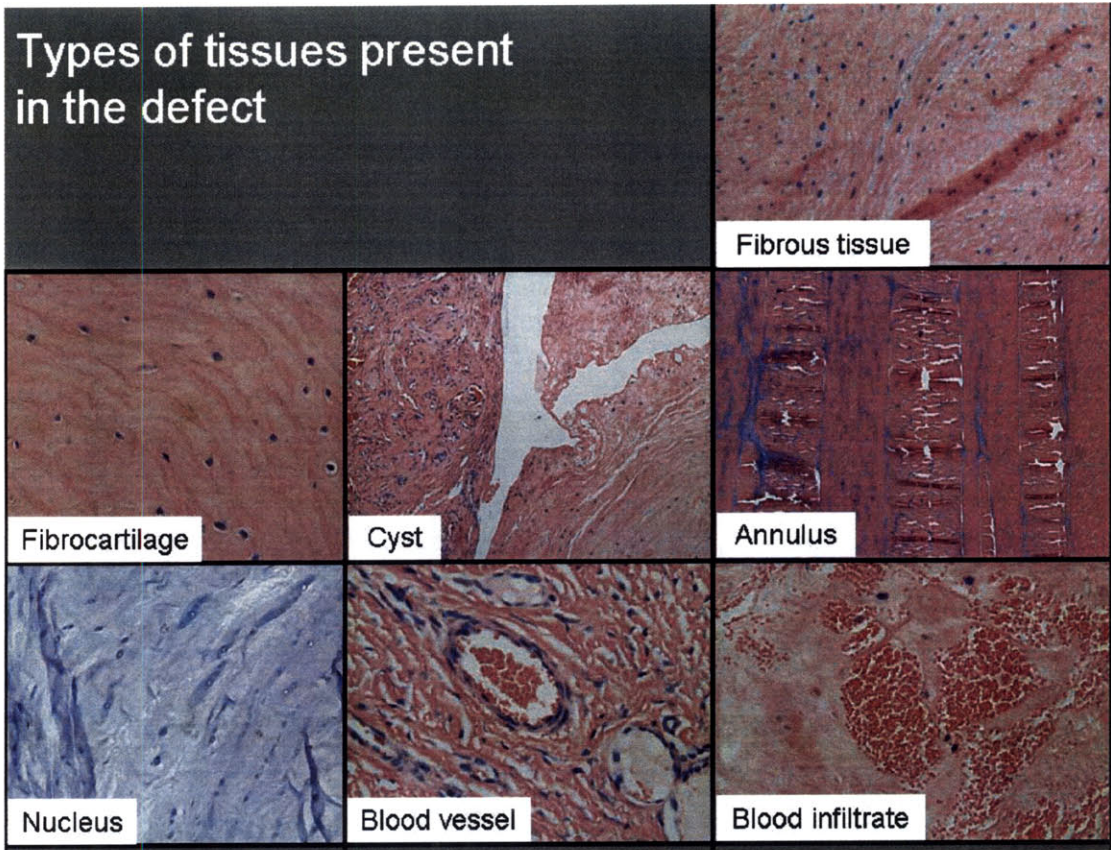
Fig. 5.15. Types of tissues present in the defect

3. Results

The surgical procedures were all successful and without any complication. Two goats were sacrificed early due to post surgical problems (pneumonia) and were replaced by two new animals. Animals fully recovered within a few hours and were neurologically intact. At sacrifice date, observation of the spine did not reveal any abnormality and scar tissue was present at the site of surgery. X-rays of the spine did not reveal any early calcification or osteophytes.
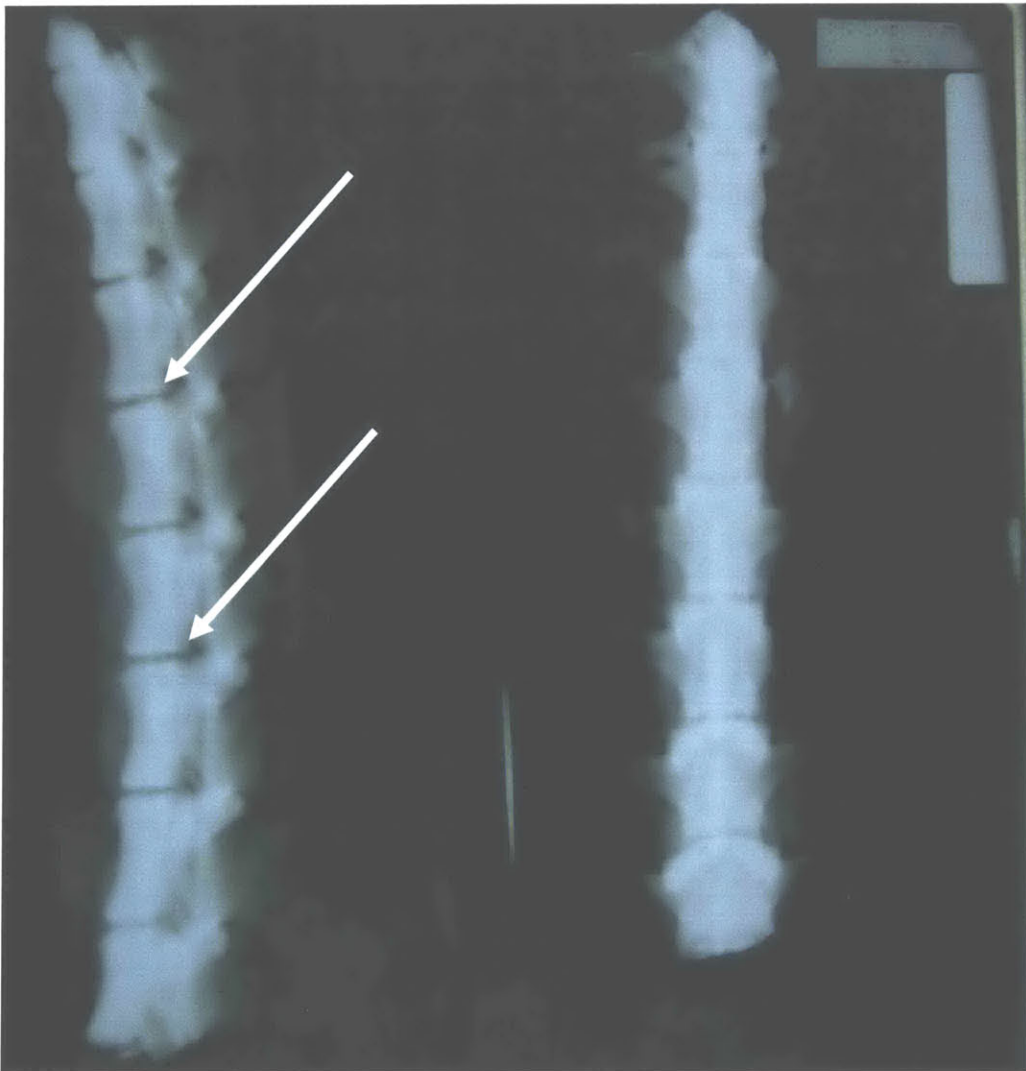
Fig. 5.16. X-ray taken post sacrifice showing a lateral and a posterior view of the spine. Operated sites (L2-L3 and L4-L5) are shown with the white arrows.

Gross morphology of the sectioned discs (see figure) showed a net separation between the annulus and the nucleus in the untouched controls and sham surgeries. In the other groups, the site of defect was easily noticeable with often times a large site of inflammation. The nucleus was often seen bulging out of the disc. At the boundary with the bone, the bone line had changed its geometry from a very curvy line to a flatter one. No other obvious difference could be noted across the other groups, except a small difference in the disc thickness.
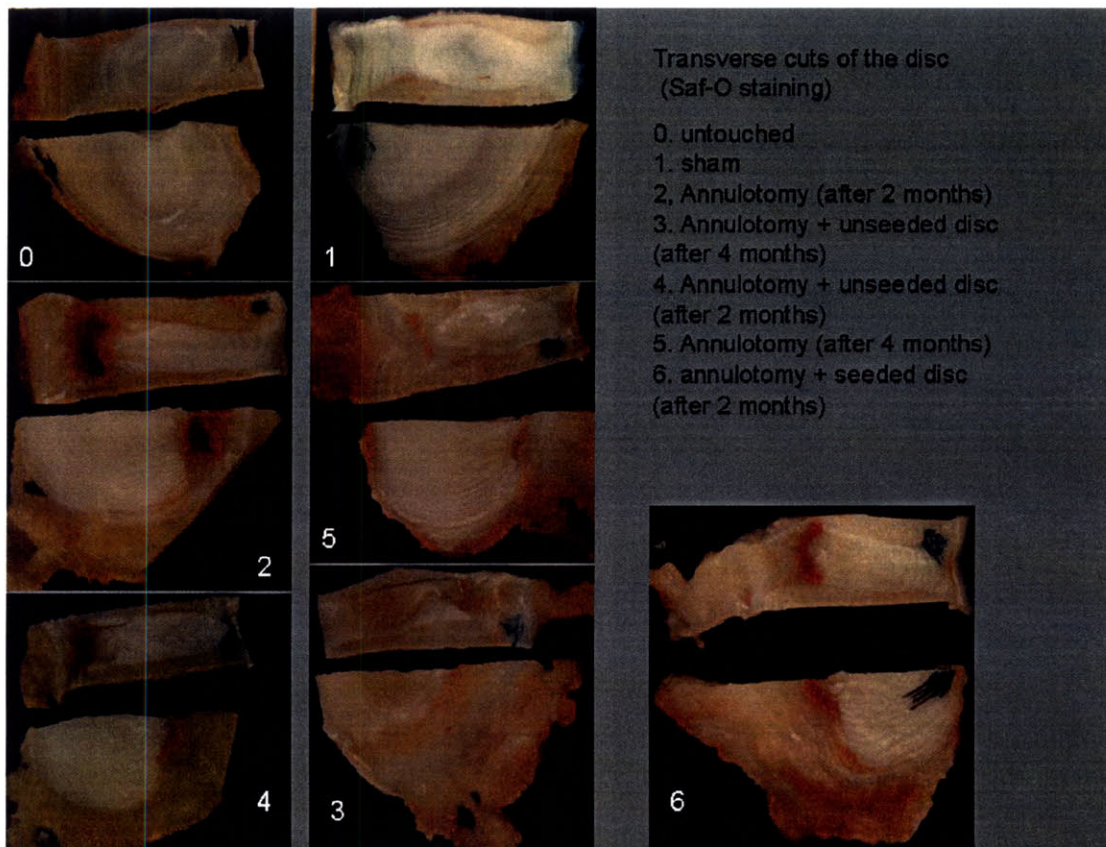
Fig. 5.17. gross morpholophy sections of the disc of the 6 different groups. (the blue spot is just a marker of the direction of the disc)
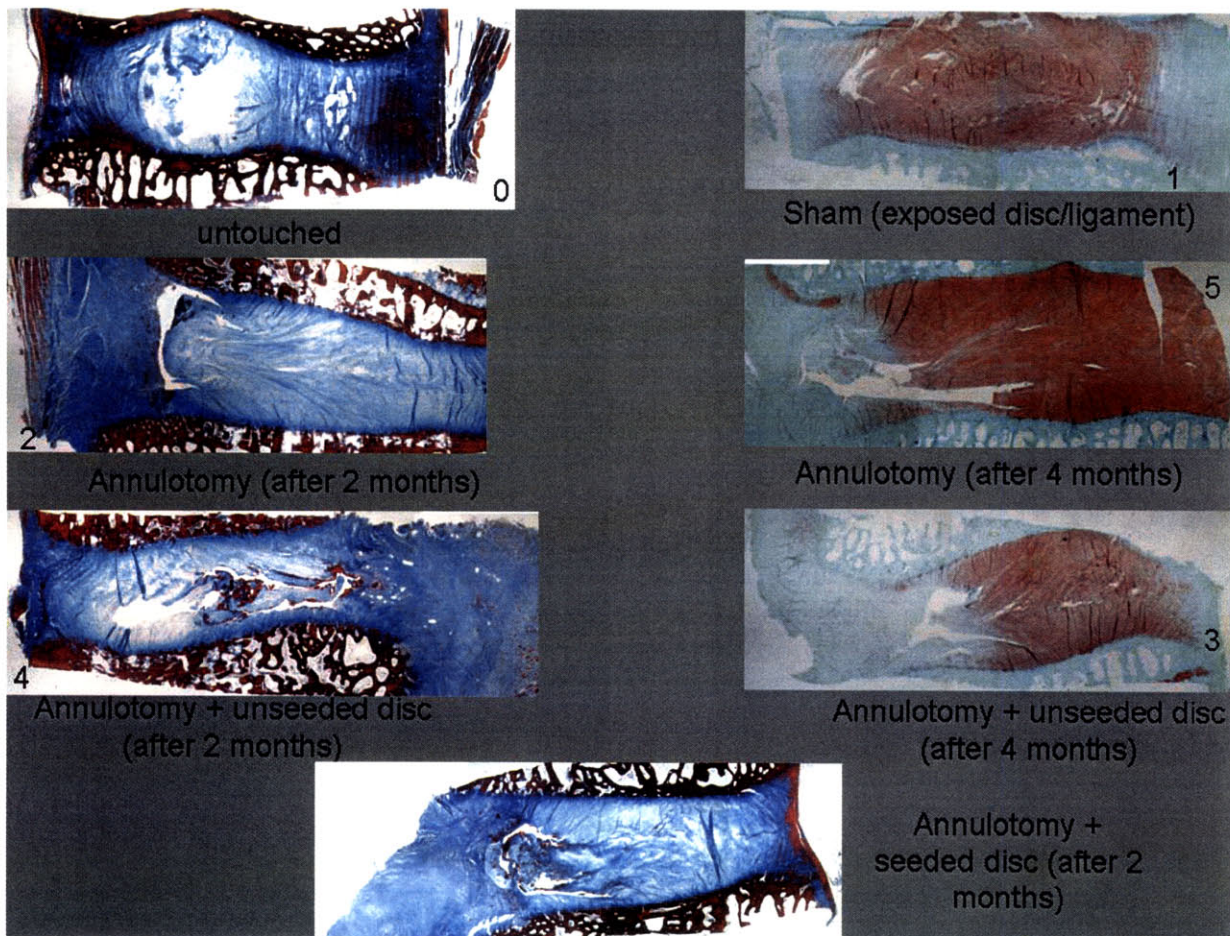
Fig. 5.18. Representative sagittal histological sections (either Saf-O or Masson) of 0) healthy untouched intervertebral disc 1) disc who has been exposed (sham), 2) disc that has undergone annulotomy through a standardized defect and sacrificed after 2 months, 3) disc that has undergone annulotomy through the same defect and sacrificed after 4 months, 4) disc that has undergone same defect in which an unseeded scaffold was placed; animal was sacrificed after 2 months, 5) disc that has undergone the same defect in which an unseeded scaffold was placed; animal sacrificed at 4 months, 6) disc that has undergone the same defect but in which an autologous annulus fibrosus cell-seeded scaffold was placed; animal sacrificed after 2 months.
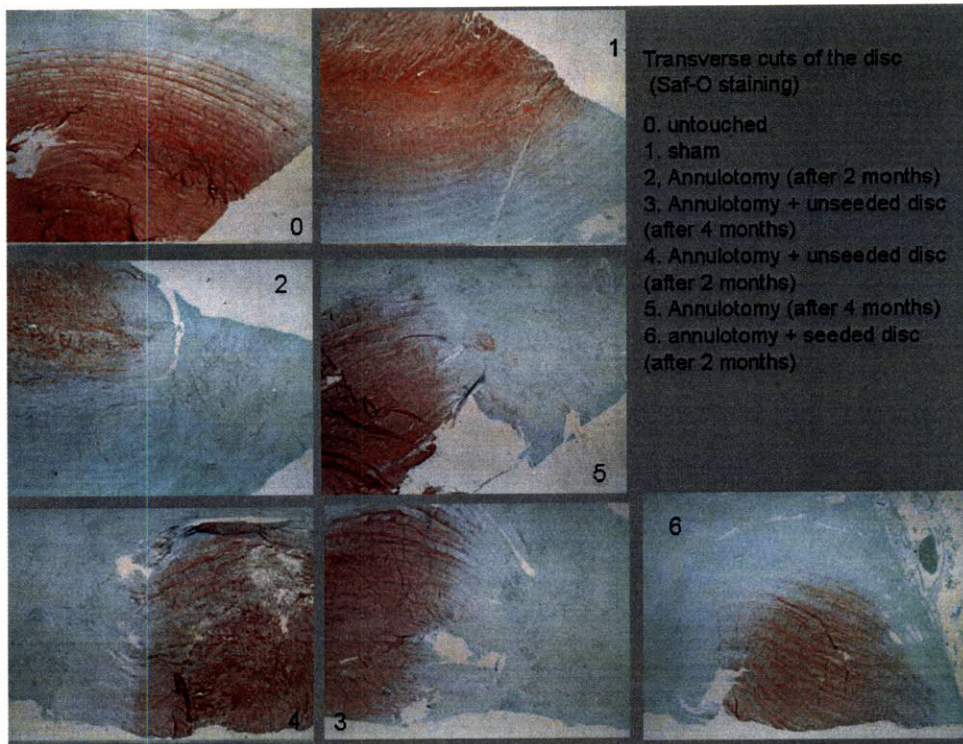
Fig. 5.19. Representative transverse histological sections (Saf-O) of the 6 groups

Histomorphometric evaluation

There was a significant effect at 2 months of the presence of the scaffold on the amount of fibrocartilage tissue ($p < 0.001$) contained in the defect but no effect of the presence of the cells in the scaffold. Untreated defects at 2 months did not contain any amount of fibrocartilage, most of the tissue filling the defect being fibrous tissue (fig 5.20)
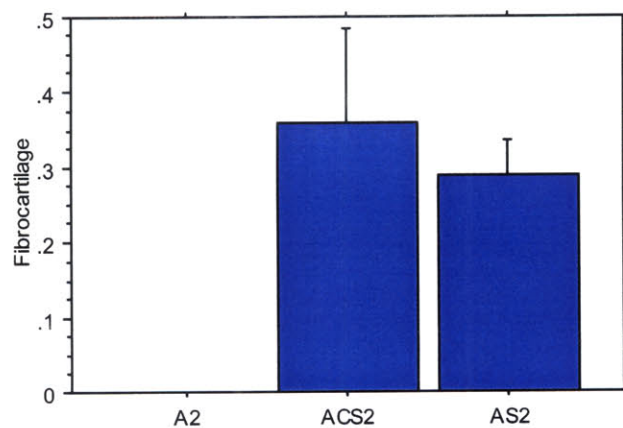
Fig. 5.20. Amount of fibrocartilage as a percent of the total region of interest for the 3 groups at 2 months: Annulotomy alone (A2), Annulotomy + scaffold (AS2), Annulotomy + cell seeded scaffold (ACS2).

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The amount of fibrous tissue at 2 months was not statistically significantly different in the same 3 groups (ANOVA, p=0.1) (Fig. 5.21).
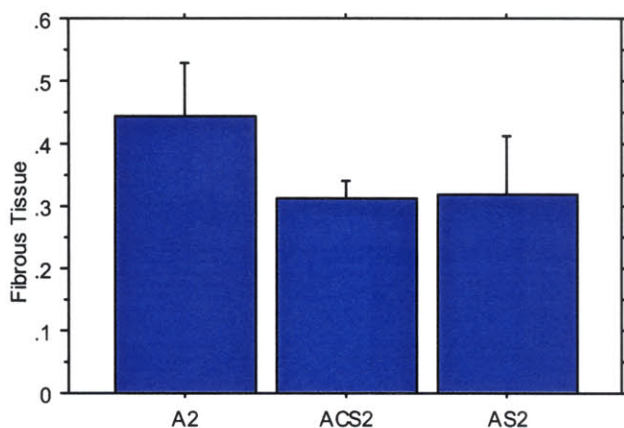
Fig. 5.21. Amount of fibrous tissue as a percent of the total Region of Interest for the 3 groups at 2 months: Annulotomy alone (A2), Annulotomy + scaffold (AS2), Annulotomy + cell seeded scaffold (ACS2).

At 4 months however, defects with scaffolds and untreated defects both presented fibrocartilage, with no statistically significant difference in their means (Fig 5.22)
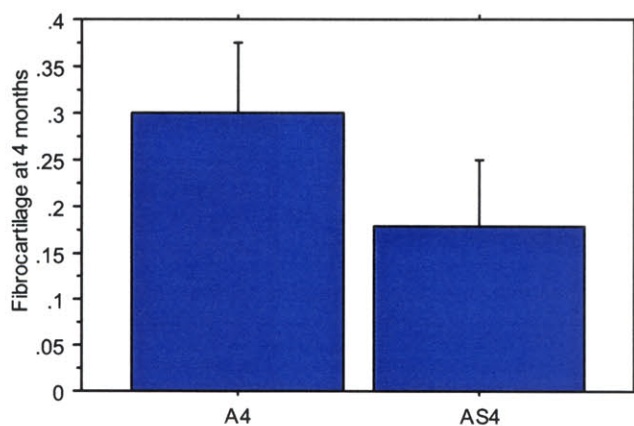
Fig. 5.22. Amount of fibrocartilage as a percent of the total Region of Interest for the 2 groups at 4 months: Annulotomy alone (A4), Annulotomy + scaffold (AS4). No statistical difference between the means was noted.

An ANOVA analysis revealed no statistically significant difference of the cyst area among all the groups (p=0.22, Fig 5.23)
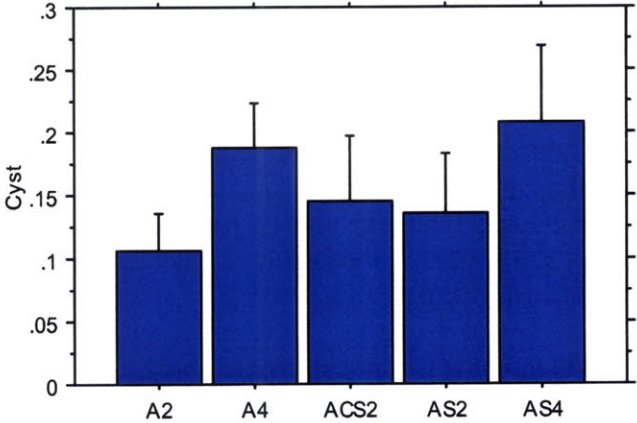
Fig. 5.23. Size of the cyst as a percent of the total Region of Interest for the 5 groups: Annulotomy alone at 2 months (A2), Annulotomy + scaffold at 2 months (AS2), Annulotomy + cell seeded scaffold at 2 months (ACS2), Annulotomy at 4 months (A4), Annulotomy + scaffold at 4 months

4. Discussion and Limitations

Of note was the higher amount of fibrocartilage in the scaffold implanted disc levels at 2 months compared to untreated controls. Fibrocartilage is the native tissue of the annulus fibrosus. At 4 months, this difference was not observed. However, at 4 months after surgery, a cell-seeded scaffold could have more fibrocartilage regeneration than an untreated control. Fibrocartilage could either originate from the cell-seeded scaffold, or from existing annulus lamellae that have lost their lamellar structure. The fact that there was a similar amount of fibrocartilage in the discs that received a cell-seeded or a scaffold alone, showed that there was little effect of the presence of the cells at 2 months. Although at 2 months, the ACS2 group had a slightly higher amount of fibrocartilage than the AS2, this was not statistically significant.

Of importance was also the fact that the biopsy procurement, cell harvest, growth and scaffold seeding as well as surgeries were all successful with no complication or technical difficulty, making the whole process of value for future medical exploration. However, it was sometimes relatively difficult to aim exactly at the disc level. This should not be as difficult during human surgery where more machines are available.

The specific hypotheses for this experiment were:
- surgical implantation is feasible: accepted. There was no difficulty to find the disc, perform the annulotomy and implant the scaffold.
- sham operation may lead to future degeneration of the disc: rejected. All sham operations did not reveal any subsequent damage or degeneration of the disc 4 months postsurgery.
- repair of the damaged disc is enhanced by the implantation of a cell-seeded collagen-GAG scaffold: accepted. At 2 months, the presence of a higher amount of fibrocartilage compared to the untreated controls shows the benefit of a cell-seeded scaffold.
- A scaffold alone induces an inferior regeneration than a cell-seeded scaffold: rejected. At 2 months, there was no significant difference on the amount of fibrocartilage present in the defect between the scaffold alone and the cell-seeded scaffolds groups.
- Scaffold will integrate into the surrounding tissue: rejected: there was no difference of the cyst size or of its integration with the surrounding tissue among all the groups.

Limitations of this premiere *in vivo* experiment into a large animal model:
- a real standardized defect should incorporate animal to animal variations, such as differences in the thickness, length and geometry of the disc. Other studies usually use a needle puncture as standardized defect. However, a disc puncture would not allow the implantation of a scaffold.
- The scaffold was already degraded at the time that the sacrifice was made, making it impossible to identify what cells were present at the time of implantation and at the time of surgery.

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- Although amount of fibrocartilage was higher in scaffold implanted levels, the mechanical aspect of the disc was not evaluated.

Future studies should:
- Create a more standardized defect for example by using special blades that would have a stopper. Standardized defects should help evaluate quantitatively the regenerative response
- Study the mechanical characteristics of the regenerated disc tissue
- Track the cells in order to see if cells have migrated in or out of the scaffold. This could be done for example with a β-galactocidase transfection.
- Evaluate the regenerative response on a longer time period
- Compare the response of annulus fibrosus cells to stem cells that are easier to be harvested.

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CHAPTER 6 – SUMMARY OF RESULTS, LIMITATIONS AND FUTURE WORK

The results of this thesis showed encouraging results *in vitro*, with an optimized culture method, which preserves cell phenotype and leads to a construct ready to implant after less than 10 days of culture. However, *in vivo* experiments still need optimization, in particular to succeed in integrating the construct with the surrounding tissue.

A summary of the specific results of each chapter is presented below.

Chapter 2 – Bioreactor study

This study revealed the advantages of using a rotating wall bioreactor compared to static culture. Indeed, the tissue constructs made from adult canine annulus fibrosus cell-seeded type II collagen-GAG scaffolds showed:
1) faster (by 2 weeks) formation of a cartilaginous construct with sufficient mechanical integrity to be handled and implanted;
2) increased proliferation of cells and mass of the constructs from 2 to 4 weeks of culture; and
3) increased biosynthetic rates and accumulated GAG over the 4-week time period of the study.

However, two main concerns prevented the use of these bioreactors in later experiments:
- high sensitivity and low replicability of the results: in a total of three experiments using the bioreactor, only one was successful in making new tissues, the other ones saw the cells be lost to the bioreactor medium.
- loss of cell phenotype: cells appear like fibroblasts and the newly synthesized tissue is a disorganized fibrous tissue mostly located on the edges of the scaffold.

Chapter 3 – Growth factors and identification of a well defined medium composition

In this study, we observed that FGF-2 and OP-1 upregulate cell proliferation and matrix synthesis, a result that was less obvious for TGF-β1. New matrix molecules were uniformly spread through the scaffolds, which was an important improvement from previous experiments (chapter 2). However, new matrix was still insufficient and cell phenotype was still unclear.

Chapter 4 – Effects of cross-linking, presence of GAG, make up of the scaffold

In this chapter, the target was to obtain as much new matrix as possible. For that, DHT, another cross-linking technique was used, in order to facilitate matrix production. Growth factors used was a mix of TGF-β1 and FGF-2, both at 10ng/mL. Results were extremely promising and showed that:

- the type of cross-linking (DHT vs. EDAC) determines new matrix production
- the make up of the scaffold (type I vs. type II collagen) has a significant effect on the type of new matrix and on the organization of the cells in the scaffold
- the presence of GAG in the scaffold is beneficial in type II collagen DHT scaffolds for the maintenance of the cells and of the new matrix
- besides, it was observed no significant difference between constructs obtained from caprine and canine cultures.

The results of this chapter showed finally maintenance of the fibrochondrytic phenotype of the AF cells in a collagen scaffold. Although the collagen orientation is still not similar to the tissue lamella orientation, we suggested that the construct could remodel *in vivo*.

Compared to previous chapters, the construct was much more uniform, contracted by just 50% after 1 week with no important additional contraction between 1 and 2 weeks. The construct appeared ready within 10 days of culture for implantation, making this process a suitable one for medical application. Also, the construct contained between 40 and 60% (in mass) of the GAG contained in a native AF tissue. One limitation is that type I collagen was not much found in the constructs and that type II collagen was preponderant. This is different from what is observed in the native tissue where type I collagen is usually more abundant than type II in the annulus.

Chapter 5 – *In vivo* evaluation of the construct in a canine model

This final chapter evaluated the response of the construct developed in the previous chapters in a standardized defect of the AF created in a lower lumbar disc of a goat. This canine model was first shown to be a suitable model for tissue engineering of the IVD, mostly because of the disc size that resembles human disc size and because of degenerative changes as the goat disc also displays degeneration.

Results showed that the repair of the disc was enhanced by the implantation of a scaffold, however, without observing any difference between cell-seeded and unseeded scaffolds. The presence of the scaffold induced lower amount of fibrous tissue after 2 months and higher amount of fibrocartilage. There was no difference between the untreated defect and the unseeded scaffold after 4 months. Scaffold did not integrate well with the defect as seen by the cyst size that wasn't different among all the groups.

Limitations of this work include the following:
- The standardized defect was hard to create: the standardized defect should incorporate animal to animal variations, such as differences in the thickness, length and geometry of the disc, which was not the case here.
- The scaffold was already completely degraded at the time that the sacrifice was made, making it impossible to identify what cells were present at the time of implantation and what cells had migrated into the defect.
- Although amount of fibrocartilage was higher in scaffold implanted levels, the mechanical aspect of the disc was not evaluated.

Conclusion and future work

The results of this study are quite encouraging, particularly when looking at the progress that was made on the *in vitro* construct and on the amount of new data gathered during the *in vivo* experiment. In the perspective of using a cell-seeded scaffold for the regeneration of the annulus fibrosus, the following is a list of future possible experiments, in order of decreasing importance and priority:

- Find a method to integrate the scaffold better in the standardized defect. This could be done by the use of a bonding agent, a gel for example,
- Create a more standardized defect for example by using special blades that would have a stopper. Standardized defects will help evaluate more quantitatively the regenerative response,
- Track the cells in order to see if cells have migrated in or out of the scaffold. This could be done for example with a β-galactocidase transfection,
- Compare the response of annulus fibrosus cells to stem cells that are easier to be harvested,
- Study the mechanical characteristics of the regenerated disc tissue,
- Evaluate the regenerative response on a longer time period.

APPENDIX

Presented here in detail are only the protocols that were changed or added since my MS thesis. For more detail, please refer to my MS thesis.

A-1 Annulus Digestion

Equipment for Annulus Digestion

- 70 μm nylon cell strainers (sterile)
- 50 mL centrifuge tubes
- complete medium (20% FBS)
- Collagenase (355 U/mg, type IA; #C9891, Sigma)
- D-PBS

Annulus Digestion

1. Enzymatic digestion must take place no longer than 4-6 hours following harvest. The annulus tissue that has been harvested and cut into small chunks of about 1mm^3 are placed in 50 mL centrifuge tubes with just enough PBS to wash the explants.
2. These are spun for 1-2 min. The PBS in removed and the tissue is rinsed twice more with PBS in the same manner.
3. The small chunks are placed in a collagenase solution containing 50 mL of DMEM/F12 and 0.15g of collagenase type I. The collagenase solution should be prepared in advance and stored at 4° C until needed.
4. The solution containing the tissue is placed in a small tissue culture flask and placed on an agitator overnight.
5. The next day, the solution is sucked out, filtered, then centrifuged. Residual tissue can be kept and cultured as well.
6. The pellet is resuspended into medium and cells are counted. After counting, the cells are resuspended at a concentration to suit culture or freezing. For culture, 2 million cells should be placed in a 75 cm^2 tissue culture flask. Complete medium containing 20% FBS should be used.

A-2 Freezing Cells

Genera1
For freezing 6 million cells in each 5 mL cryogenic tube, or 3 million cells in a 3mL cryogenic tube.

Materials
Complete Medium
DMSO (Dimethyl sulfoxide). Make sure it is sterile before use (can use syringe filter for that purpose)
Sterile Filter
Sterile cryogenic tubes
Freezing box

Methods
1.   Determine amount of medium needed (1 mL per 2×10^6 cells) and add it to cells in 50 mL centrifuge tube.

2.   Add 10% DMSO (i.e., if there is 15 mL of cell/medium suspension in 50 mL tube, add 1.5 mL of DMSO.)

3.   Quickly place in cryogenic tubes (3.3 ml per 5 ml tube) and freeze immediately in the freezing box (it controls freezing rate) at -80C. The next day, place in liquid nitrogen

A-3 Thawing Cells

This is a fast process. Cells should be placed in warm medium as fast as possible after thawing.

Materials
Warm complete (20% FBS) medium
Tissue culture flasks

Methods
1.   Preheat medium. Place cryogenic tubes directly into a 37°C water bath. Agitate gently while cells thaw for 60 seconds.

2.   Spray the tubes with alcohol and transfer them into the hood. Slowly add a few drops of warm medium to the tube until it is full. Recap the tubes and agitate until completely thawed.

3.   Transfer the cells to a 50 mL tube and add more warm medium. Wash them clean of medium + DMSO for 10 minutes in the centrifuge.

5.   Count the cells, and resuspend at the proper concentration. For 75 cm^2 tissue culture flasks, the thawed annulus cells should be plated at 1 million cells per flask. Cells should be cultured at least 3-4 days before being used for experimentation (or before changing medium, depending on when they attach).

A-4 Passaging Cells

Please refer to my MS thesis

A-5 Cell counting

Please refer to my MS thesis

A-6 Medium preparation

Please refer to my MS thesis

A-7 Medium changing

Please refer to my MS thesis

A-8 Scaffold Making

Type I Collagen – (from Hastreiter)

<u>Slurry making</u>

1. Cool blenders to 4° C (takes at least 30 min.) using directions on the wall. Steps 2-4 and 6 can be done while waiting. Step 1 of the freeze-drying protocol should also be performed if freeze-drying immediately.

2. Prepare 0.05 M acetic acid if unavailable:
17.4 ml glacial acetic acid + enough distilled water to make 6 L = 6 L of 0.05 M acetic acid
Glacial acid is in the cabinet across from the blenders labeled "acids."

3. Fill the blender with 600 ml of 0.05 M acetic acid. One blender gives enough slurry for 3 sections of 1 freeze-dryer tray.

4. Weigh 3.6 g of dry tendon collagen (kept in the refrigerator). Use right scale.

5. Place collagen in the blender and blend on high for 90 min.

6. Mix 0.32 g chondroitin 6-sulfate (in dessicator in the refrigerator) in 120 ml of 0.05 M acetic acid with magnetic stirrer. Use the left scale for weighing.

7. Add 120 ml of chondroitin 6-sulfate solution over 15 min using the peristaltic pump. Make sure the switch is on "reverse."

8. After the addition, blend the mixture for an additional 90 min. on high.

9. Pour out slurry and refrigerate if not freeze-drying immediately. Slurry can be used for up to one month after making. If longer than a week after making, reblend for 15-30 min. Clean the blender with 0.05 M acetic acid.

10. De-gas the slurry with a vacuum flask for 10-30 min. (latter time for the current machine). Clean the vacuum flask afterwards.

<u>Vitreous Freeze-drying Protocol</u>

1. Drain condenser (tube under condenser). Turn on the freeze button and the condenser button.

2. Wait for shelf to cool down to -45° C (at least 1 hour).

3. Clean the freeze-drying tray with 0.05 M acetic acid. Put the amount of slurry into the long trays based on the thickness you desire below. Avoid bubbles as much as possible and try to pop the ones that form.

"*skin protocol*:" Pour the slurry into the tray if using a pan with one section. If using a pan with 3 sections, pipette the slurry into the sections in equal amounts.

"*1/2 thickness*:" Pipette the slurry into the sections of 2 whole trays (6 sections) in equal amounts.

"*double thickness*:" Pour all the slurry into the half width unsectioned tray.

"*cartilage protocol*:" Pipette 180 mL of slurry per section of a tri-partitioned tray.

4. Wait for approximately 1 hour until the slurry is frozen (or more if it doesn't look frozen). (For a half tray it takes about 1.5 hours.)

5. After the slurry is frozen, turn on the vacuum. First, make sure the chamber release button is off. Once vacuum is on, press door shut. Make sure the door is sealed before leaving. Often the door will not seal and the vacuum will never establish itself - this is not good for the vacuum pump!

6. Once the vacuum is below 200 mtorr (0.5-3 hours depending on the ambient conditions and when the freeze-dryer was last serviced), turn the temperature set to 0° C. Leave both freeze and heat buttons on. Turn on heat button if not previously on. Leave overnight or at least 12 hours for sublimation.

7. Set temperature to 20° C and turn off freeze button. (Leave on heat button.)

8. Turn the DHT temperature setting to slightly past 105° C if DHTing immediately.

9. When the freezer is at 20° C, turn off the heat button, vacuum button, and condenser button. Release the chamber. Remember to drain the condenser chamber. After defrosting, the chamber and condenser should be wiped dry with a paper towel. Don't forget to place the plug back in the drain for the next run.

DHT Cross-linking

After freeze-drying, the thickness of the matrix should measured with a micrometer. Then, the matrix should be placed into the DHT oven for DHT cross-linking for 24 hours. The conditions of the vacuum oven are 1 atm and 105° C. The matrix sheets are placed in aluminum foil with one end open. Additionally, this can be placed in a tape-sealed autoclave bag for added sterilely when you remove the matrix but it is unclear how this affects the cross-linking. Be careful not to crumple the edges of the matrix sheet. After 24 hours, the matrix should be stored in a dessicator prior to use. The instructions for starting the vacuum on the DHT and purging the oven are on it.

Storage
Unless hydrated, all matrices should be stored in a dessicator with blue desiccant.

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<u>C-8-1-b temperature ramping protocol</u>
Basically, the protocol is the same as above. Smaller pans are used so that only 67mL of slurry is used to fill the pan and have the same thickness. Freezing of the slurry is done using a temperature ramping programmed on the machine: temperature decrease is done from 20°C to -50°C in 2 hours.

Type II Collagen scaffold
- Reconstitute type II collagen slurry by mixing 1g of collagen type II for each 100mL of 0.05M acetic acid. Use the blender set on high. Mix for 5 minutes.
- Keep blenders cold during the operation
- If making collagen-GAG scaffolds, add 5% (ie. 0.05g for each gram of collagen) or 10% (ie. 0.1g for each gram of collagen) of GAG and continue blending.
- Pour slurry into the plastic trays and free dry using the standard temperature ramping protocol.
- DHT scaffolds after production if necessary

A-9 Cell seeding using pipetting method

This « one side dry scaffold » method simplifies the once used "two sided and prewet scaffold" method. It leads to higher consistency of the results and better cell spreading in the scaffolds. It is only possible with DHT cross-linked scaffolds (that are already dry).

1. DHT cross-linked matrices should be seeded dry and on one side only. First, the optimal amount of medium to be used should be found by trials and errors: for that, add a little bit of medium onto a dry scaffold and increase that amount on different scaffolds. Wait for about 5 to 10 minutes and find which amount of medium optimally saturates the scaffold with medium.

2. To seed, the cells are first passaged from the flasks. They should then be suspended at the desired concentration (usually around 50 million cells / mL).

3. The cells are then added on top of the scaffold on the side with the large pores. If the diameter of the scaffold is large, it may be beneficial to seed 3 drops of medium/cells onto the scaffold.

5. Placed the matrices in an incubator for 2 hours. Then add around 0.5mL of medium (serum free or with FBS, following what is needed) to each well very slowly and along the sides of the wells. This is to prevent cells from being pushed out of the scaffold. This amount should just be enough to cover the bottom of the well with medium.

6. Incubate overnight. The next day, fill the well with medium (for a total of 3mL for a 6 well plate for example)

A-10 EDAC Cross-Linking
Please refer to my MS thesis

A-11 Matrix culture in multi well-plates
Please refer to my MS thesis

A-12 Radiolabeling of 35S and 3H
Please refer to my MS thesis

A-13 Lyophilization of Matrix Samples
Please refer to my MS thesis

A-14 Papain digestion
Please refer to my MS thesis

A-15 Scintillation counting of 35S and 3H Radiolabeled Samples
Please refer to my MS thesis

A-16 GAG Assay (Dimethylene Blue)
Please refer to my MS thesis

A-17 DNA Assay using Hoechst Dye
Please refer to my MS thesis

A-18 Paraffin embedding of Matrices
Please refer to my MS thesis

A-19 Preparation of coated slides
Please refer to my MS thesis

A-20 Hematoxylin and Eosin Staining
Please refer to my MS thesis

A-21 Safranin-O Staining
Please refer to my MS thesis

A-22 Masson Trichrome Staining
Please refer to my MS thesis

A-23 Type II immunohistochemistry
Please refer to my MS thesis

A-24 Protocol to dissect the spines

prepare blades #10, blade holders #3, pick up forceps, chisel + hammer

1. take X-ray of the spine
2. locate the discs on the x-ray and label them
3. the small discs (short vertebrae) are often in the superior position.
4. put the spine in formalin for one night
5. clean the spine by removing the muscles on the posterior side (the one that was not operated on)
6. The anterior side is the one that was operated on, L2-L3 was operated on the left side, L4-L5 on the right side. To identify these areas, place the spine on the bench, with the inferior side towards you and the superior side far from you. The spinous process must be on top and the operated side is touching the benchtop. This is the natural position of the spine in the animal: the L2-L3 operated site will be the one on the left and the L4-L5 will be the right operated site.
7. locate the operated sites very carefully
8. use the saw to cut through the adjacent discs
9. use the saw to cut off the transverse processes, the spinous process
10. cut through the pedicle to obtain just a disc with its two endplates.
11. cut the adjacent vertebral bodies to obtain as thin as possible a disc + two end plates.

DURING ALL THE PROCESS, MAKE SURE YOU HAVE LOCATED THE OPERATED SITE.

12. Mark the superior side with the saw
13. place the discs in formalin (10x the volume of the discs) for 1week
14. start the decalcification process. Replace the EDTA solution every 3 days.
15. after a few days, cut approximately through the defect sagitally , and continue the decalcification until bone is soft enough to be cut. Try to trim the bone a little bit more until it's very thin.