DISSERTATION

HEALING OF FULL THICKNESS CHONDRAL DEFECTS TREATED WITH ARTHROSCOPIC SUBCHONDRAL BONE PLATE MICROFRACTURE AND IL-1RA / IGF-1 DELIVERED THROUGH GENE TRANSFER

SUBMITTED BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SOPHIE MORISSET ENTITLED "HEALING OF FULL THICKNESS CHONDRAL DEFECTS WITH ARTHROSCOPIC SUBCHONDRAL BONE PLATE MICROFRACTURE AND USING IL-1RA AND IGF-1 DELIVERED THROUGH GENE TRANSFER" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

COMMITTEE ON GRADUATE WORK

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ABSTRACT OF DISSERTATION

HEALING OF FULL THICKNESS CHONDRAL DEFECTS TREATED WITH ARTHROSCOPIC SUBCHONDRAL BONE PLATE MICROFRACTURE AND IL-1RA / IGF-1 DELIVERED THROUGH GENE TRANSFER

Hyaline articular cartilage is a specialized tissue that needs an intact structure to perform its physiologic functions. Once damaged, cartilage typically heals with fibrocartilage and fibrous tissue. These tissues do not possess the biomechanical and biochemical properties of the original hyaline cartilage, and therefore compromise the integrity of the articular surface and affect normal joint function.

Various techniques have attempted to improve healing of cartilage defects. So far, no single method has clearly shown advantages over the others. Previous work in our laboratory has that a technique; arthroscopic subchondral bone plate microfracture (SBPM) increases the amount of repair tissue present in the defect and improved the quality of cartilage repair by increasing the amount of type II collagen but, was unable to up regulate the synthesis of proteoglycans.

This project was performed to evaluate if the intra-articular injection of adenoviral vectors carrying the equine genes of interleukin-1 receptor antagonist (IL-1ra) and insulin-like growth factor-I (IGF-1) would enhance the healing of experimentally created cartilage defects treated with SBPM. Using an equine model of full thickness chondral

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defect the effects of gene transfer of AdEqIL-1ra and AdEqIGF-1 on cartilage healing was evaluated. Results of the study demonstrated an up-regulation of IL-1ra expression for a period of 21 days and an increased endogenous production of IGF-1 for a period greater than six weeks. These increases in protein expression were associated with improvements in the biochemical composition of the repair tissue. Repair tissue in defects of treated joints showed an increased amount of proteoglycans and type II collagen content as demonstrated by biochemical and immunohistological analyses.

This study was able to show that gene transfer of an anabolic growth factor and an anti-inflammatory molecule can successfully be used to enhance cartilage healing in the horse and will hopefully bring us a step closer to effectively modulate the production of long lasting functional repair tissue.

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I also have to thank all the volunteers that gave up their study time to come and help with the horses. And of course I cannot forget the "S horses", S1 = Squeakers, S2 = Goliath, S4 = Athena, S5 = Withy, S6 = Speckles, S7 = Galaxy, S8 = Zoe, S9 = Peaches, S10 = Chevy, S11 = Winston, S12 = Roy and S14 = Spook

Finally I have to give a very special thank you to my parents Jean et Claudine that gave me the strength and determination to always pursue my dreams.

Thank you everybody.

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PREFACE

For centuries man has known about the poor healing capacities of articular cartilage. For centuries scientists have been trying to understand the physiology of cartilage healing and find a way to intervene in the process to generate repair tissue that would possess the biochemical and biomechanical quality of the original articular cartilage.

Throughout the years science has been able to decipher many of the components of the mechanisms involved in the degradation and repair of articular cartilage. This knowledge has been the basis of many treatment modalities created with the objective of enhancing cartilage healing. Some methods were more successful than others and are presently being used in clinical cases. However none has attained the ultimate goal of generating long lasting functional repair tissue.

The work presented here is an attempt to combine the use of gene therapy to a technique proven effective in the treatment of chondral lesions. We hope that the combination of the two techniques will yield a repair tissue of greater quality bringing us a step closer to solving the problem of cartilage healing.

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CHAPTER 1

1 Introduction

1.1 Purpose

The purpose of this study was to determine if gene therapy would enhance the effects of subchondral bone plate microfracture (SBPM) on the healing of full thickness chondral defects created on the distal weight bearing surface of the radial carpal bone and the medial femoral condyle.

1.2 Goals

The goal of this study was to investigate the therapeutic effects of the anabolic growth factor insulin growth factor-1 (IGF-1) and the anti-inflammatory protein interleukin-1 receptor antagonist (IL-1ra) delivered to the joints by gene transfer on the healing of full thickness chondral defects treated with SBPM.

1.3 Statement of Hypothesis

The working hypothesis for this research project was: the combined anabolic properties of IGF-1 and anti-inflammatory effects of IL-1ra will significantly improve the quality and the quantity of the repair tissue found in full thickness chondral defects treated with SBPM, improving the clinical parameters of joint disease and reducing synovial joint inflammation.

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1.4 Specific Aims

1. To determine the therapeutic effects of the combined use of IL-1ra and IGF-1 on the clinical parameters of joint disease such as lameness and radiographic signs of osteoarthritis in femoral and carpal joints with full thickness chondral defects treated with SBPM followed by 16 weeks of controlled exercise.

2. To determine the concentration and length of expression of IL-1ra and IGF-1 in the synovial fluid of middle carpal joints with full thickness chondral defects treated with SBPM during 16 weeks of controlled exercise.

3. To determine the therapeutic effects of the combined use of IL-1ra and IGF-1 on synovial fluid parameters of inflammation in middle carpal joints with full thickness chondral defects treated with SBPM during 16 weeks of controlled exercise.

4. To determine the therapeutic effects of the combined use of IL-1ra and IGF-1 on the macroscopic appearance of the repair tissue present in full thickness carpal and femoral chondral defects treated with SBPM followed by 16 weeks of controlled exercise.

5. To determine the therapeutic effects of the combined use of IL-1ra and IGF-1 on the histologic, histochemical and biochemical characteristics of the repair tissue present in carpal and femoral chondral defects treated with SBPM followed by 16 weeks of controlled exercise.

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1.5 Background

1.5.1 Clinical importance of joint diseases

A national survey conducted by the Unites State Department of Agriculture (USDA) in conjunction with the National Animal Health Monitoring System (NAHMS) identified lameness has the most often diagnosed health problem in the United States horse population with an incidence of 8.5 to 13.7 events / 100 horses. The cost to the horse industry in 1998 was estimated to be close to $1 billion with the greater percentage (66%) associated to loss of use. [1] The same study reported that 50% of the horse operations had one or more lame horse in 1 year period and that approximately 50% of those lamenesses could be associated to leg or joint problems. [2] These results are similar to those from Cornell University where 42% of the lame horses presented at the New York State College of Veterinary Medicine had lameness related to joint disease. [3] A prospective study evaluating the prevalence of osteoarthritis in the distal tarsal joints of mature Icelandic horses showed that 30.3% of the horses examined had radiographic evidence of the disease. [4]

In human, osteoarthritis (OA) and to a lesser degree rheumatoid arthritis is the most prevalent cause of chronic disease in the United States [5] and, second to heart disease for long-term paid disability. [6] It affects one in six Americans or approximately 20 million individuals [7] and, 80% of the population older than 75 years is symptomatic in at least one joint. [8] The cost to the U.S. economy was approximated at $ 65 billion in 1997 [5] and is estimated to rise significantly in the next 15 years. Effectively predictions from the Center for Disease Control and Prevention evaluate that by the year 2020, OA will have the largest increase in new number of patients of any disease in the United States. [9]

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Because of this significant socio-economic burden in both the human and equine populations, the need to pursue basic and clinical research in the treatment and prevention of the disease becomes obvious.

1.5.2 The chondrocyte

Articular cartilage is the connective tissue [10] that covers the ends of the bones of diarthrodial (synovial) joints. [11] It is avascular, aneural and alymphatic. [11] It is composed of one cell population, the chondrocytes within extracellular matrix (matrix macromolecule frame work). [10]

The chondrocyte is a highly specialized, metabolically active cell. Its glycolic rate per cell is similar to that of vascularized tissue [10] although its metabolic activity varies depending on its location in the cartilage and decreases with age. [10] Chondrocytes represent 1 to 5 % of the volume of cartilage in human adults, [10, 12, 13]. This proportion is increased in fetal and young immature cartilage [12] and decreases with aging. [14] On the basis of weight, chondrocytes compose between 5 and 10% of the cartilage wet weight [15, 16]

1.5.2.1 Intracellular organelles and cytoskeleton of the chondrocyte

Chondrocytes are no different than any other metabolically active cell. They possess all the organelles necessary to support matrix synthesis and depending on their level of metabolic activity those organelles are more or less developed. The nucleus, the endoplasmic reticulum, Golgi apparatus and secretory vesicles are probably the most important organelles. [10] Another intracellular component of the chondrocyte is the cytoskeleton. This compartment, very often ignored, seems to play an important role in mediating chondrocyte metabolic activities. [17] Microfilaments made of polymers of β actin subunits are important in maintaining the chondrocyte phenotype. Microtubules

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made of α and β tubulins play a role in the synthesis and secretion of collagens and proteoglycans, via the intracellular transport of secretory vesicles. The intermediate filaments vimentin and cytokeratins may be involved in mediating changes in matrix component synthesis in response to mechanical loading. [17]

1.5.2.2 Role of the chondrocyte

The chondrocyte is the functional unit of articular cartilage. It is responsible for the production, organization and maintenance of the extracellular matrix via the action of anabolic and catabolic pathways. [11]

1.5.3 Structural organization of articular cartilage

1.5.3.1 Zones

Articular cartilage is composed of four major layers or zones. They are the superficial zone, the transitional or intermediate zone, the radial or deep zone and the zone of calcified cartilage. The relative thickness and appearance of each zone varies among species and among joints within the same species. [10] Although the boundary between each zone cannot be sharply defined we know that each zone has its specific extracellular matrix composition, cellular morphology and cellular metabolic activity. This organization possibly reflects a functional role where each zone responds differently to mechanical loading. [10]

1.5.3.1.1 Superficial Zone

The superficial zone is the thinnest zone of the articular cartilage. [10] It is situated at the free surface of the articular cartilage and is in direct contact with the synovial fluid. [18] It is divided into 2 layers. The most superficial layer, the lamina splendens or clear film, is acellular, devoid of proteoglycans and composed of fine collagen fibrils. [19, 20, 21] Underneath that first thin sheet of fibers there is a layer of

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small, flattened, ellipsoid-shaped chondrocytes with their major axis aligned parallel to the articular surface. These cells synthesize a matrix of dense, thin collagen fibrils that organize themselves parallel to the articular surface. [10, 18] The cells of the superficial layer are the only ones capable of synthesizing lubricin, a protein thought to play an important role in providing articulations with almost frictionless motion. [22, 23] Compared to the other zones, the superficial zone has the smallest diameter collagen fibrils (approximately 20nm in diameter), [18] the lowest concentration of proteoglycans and the highest concentration of collagen. [10] Fibromodulin is also found in the superficial layer. It is believed to protect type II collagen from enzymatic degradation and prevent cell adhesion to the cartilage surface. [24]

The role of the superficial layer is two fold. The organization of its matrix gives the superficial layer the greatest tensile strength [25, 26] and allows cartilage to resist shear compressive and tensile forces generated during motion. [27] The dense organization of the collagen fibrils is believed to create a barrier that isolates cartilage from the immune system and inflammatory molecules [10] and may be the first line of protection against osteoarthritis. [10]

1.5.3.1.2 Transitonal / intermediate zone

The transitional zone is situated directly beneath the superficial layer. The chondrocytes of this layer assume a spheroid shape and are randomly distributed throughout the matrix. [28] They are more active metabolically than those in the superficial zone active supporting a higher concentration of synthetic organelles. [10] The matrix they synthesize possess larger diameter collagen fibrils and more proteoglycans than those of the superficial layer. [10, 18] The orientation of the collagen fibrils in that layer is controversial. Some studies argue that the fibrils are arranged at right angles to the superficial layer. [29] Others suggest that the fibrils are vertical deep

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in the layer and become horizontal and parallel to those of the superficial layer as they approach the surface. [20] But the most common conception is that the orientation of the collagen fibers is random and creates a fibrous mesh. [21, 30]

1.5.3.1.3 Radial / deep zone

The radial zone is the thickest of all the cartilage zones. [28] The chondrocytes are large and round and aligned in columns perpendicular to the articular surface. [28] These cells are the most metabolically active, [28] they synthesize a matrix with the highest concentration of proteoglycans, the lowest concentration of collagen but with fibrils of the largest diameter. (70 to 120 nm). [10, 18] The collagen fibrils are also arranged perpendicular to the surface and are firmly anchored to the tidemark.

1.5.3.1.4 Tidemark

The tidemark is a thin undulating line of densely packed collagen fibril bundles delineating the boundary between the calcified and the non-calcified cartilage. It provides a smooth transition between the compliant non-calcified cartilage and the rigid calcified cartilage and subchondral bone. It helps distribute loads across the entire chondral-subchondral bone boundary, reducing tensile stress within the fibers. [21, 31, 32] The tidemark is not continuous. It is periodically interrupted by small gaps that may provide a pathway for nutrient transport. [33]

1.5.3.1.5 Calcified cartilage layer

The calcified cartilage layer (CCL) is bounded on one side by the subchondral bone and on the other by the tidemark. Its thickness averages 5% of the total articular cartilage thickness. [34, 35] The chondrocytes located in this layer are round but smaller than those in the radial zone. They have the lowest level of metabolic activity [10] and do not synthesize proteoglycans. [11] They are unique as they are the only ones

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capable of synthesizing type X collagen and calcifying the extracellular matrix. [18, 36, 37] The CCL is also unique for the fact that it is the only cartilage layer that is vascularized, a characteristic essential to control calcification and remodeling. [38, 39] The calcified cartilage/subchondral bone interface is highly irregular and held together by interdigitations but none of the collagen fibers from the CCL penetrates the subchondral bone. [37] This irregular interface plays a role in converting shear forces in the joint to compressive and tensile forces in the underlying bone. [40] The tidemark/calcified cartilage interface fibers are thought to continue through the tide mark to the superficial layer of cartilage. [41, 21] This particular arrangement of fibers is responsible for the attachment of articular cartilage to bone. [42, 43]

1.5.3.2 Matrix regions

Within each zone the chondrocytes are surrounded by an extracellular matrix that is organized in three distinct regions

1.5.3.2.1 Pericellular region

The pericellular region is a thin, narrow region with a thickness of approximately 2 μm that immediately surrounds the chondrocyte. [18] It has little or no fibrillar collagen but is rich in the non-fibrillar type VI collagen, and the cell-membrane-associated proteoglycans anchorin CII [44, 45] decorin and aggrecan. [29, 46, 47] Cytoplasmic extensions from the chondrocytes project into and through the pericellular matrix to the territorial matrix. The combination of a chondrocyte and its pericellular region is called a chondron. [11]

1.5.3.2.2 Territorial region

An envelope of territorial matrix surrounds the pericellular region of individual, pairs or clusters of chondrocytes. In the radial zone the territorial matrix surrounds the

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column of chondrocytes. [10] This region possesses thin collagen fibrils with the ones nearest to the cells appearing to adhere to the pericellular matrix. Further away from the cells the collagen fibrils seems to form a fibrillar basket around the cells. The role of the pericellular and territorial region seems to be directly associated with the chondrocytes. They bind the cell membrane of the chondrocyte to the matrix macromolecules and protect the cells from damage during loading and deformation of the tissue. [10]They may also have a role in transmitting mechanical signals to the cells when the joints deform during loading. [10]

1.5.3.2.3 Interterritorial region

The interterritorial matrix is the largest of the matrix regions. It possesses the largest collagen fibrils which are not organized to surround the chondrocytes. Their organization and orientation varies in relation to the cartilage zones. It is responsible for the mechanical properties of the cartilage. [10]

1.5.4 Extracellular matrix

The extracellular matrix of articular cartilage consists of two components: the tissue fluid and the framework of structural macromolecules. This matrix and its particular organization give the cartilage its form and stability. The interaction between the tissue fluid and the matrix macromolecules provides cartilage with is mechanical properties of stiffness and resilience [48, 49]

1.5.4.1 Tissue Fluid

Water and dissolved electrolytes compose the tissue fluid of the extracellular matrix. Water content is at it highest during development; it corresponds to 80% of articular cartilage wet weight in neonates and, decreases to approximately 65 – 70 % in normal adult cartilage. [10, 16, 50] Water content of articular cartilage also varies with

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cartilage zones, 65% of wet weight in the deep zone and up to 80% in the superficial zone. [15] Dissolved in the water are small proteins, metabolites and a high concentration of cations. [10]

1.5.4.2 Collagens

The extracellular matrix of articular cartilage is composed of type II, VI, IX, X, XI, XII, and XIV collagens. [18, 51] They contribute to approximately 60% of its dry weight. [49]

1.5.4.2.1 Type II collagen

Type II collagen is the most abundant type of collagen in articular cartilage accounting for 90-95% of all collagens. [18, 52] It is also the most important component of the collagen fibril, composing approximately 90% of each. [18] Like all types of fibrillar collagen, type II collagen is made of three α-chains characterized by a Gly- X-Y sequence, where X is frequently a proline and Y a hydroxyproline. [53] In type II collagen these three chains are identical, forming the homotrimeric molecule $[\alpha_1 (II)]_3$ [53] encoded by the gene COL2AI. The equine type II procollagen mRNA has been cloned. It is 4.3 kb and is 92.4% homologous to the human sequence. [54] The equine propeptide consists of 1418 amino acids with a calculated molecular weight of 134 kDa and its sequence is 97% identical to the human propeptide. [54] Type II collagen is responsible for the tensile strength of cartilage. [10, 16, 55] The α chains of type II collagen are synthesized in the rough endoplasmic reticulum (RER). Within the RER the α chains associate to form the triple helical procollagen molecule consisting of a helical domain, and non-helical C^- and N^- propeptide domains. This association is made possible by the formation of disulfide bonds between cysteine residues present on adjacent chains. [53] The procollagen molecules are packaged in secretory vesicles by the Golgi apparatus and are secreted in the extracellular space. It is there that the

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procollagen is converted to collagen by the action of specific enzymes that cleave the C^- and N^- propetides. The mature collagen molecule also called tropocollagen consist of a main triple helical domain made of the three identical α chains (α_1(II)) and of two short non-helical flanking domains called C^- and N^- telopeptides. Tropocollagen molecules further assemble into fibrils stabilized by aldol condensation and intermolecular covalent hydroxypyridinum cross-links between lysine and hydroxylysine residues of adjacent molecules giving the fibril its high breaking strength. [56] Each fibril is composed of approximately 50 to 100 collagen molecules and has an approximate length of 300nm.

1.5.4.2.2 Type IX collagen [57, 58, 59]

Type IX composes approximately 3% of the total collagen of articular cartilage. It is an heterotrimeric molecule composed of 3 different α chains [α_1(IX)α_2(IX)α_3(IX)] characterized by two short non-helical domains that interrupt the triple helix and a large globular non-helical N-terminus domain called NC4. The interruption of the triple helix causes a kink in the molecule where a chondroitin sulfate rich proteoglycan may attach itself on the α_2(IX) placing the molecule in the category of proteoglycans. [60, 61] It is one of the three types of collagen composing the collagen fibril. It is located at the exterior of the fibril in an antiparallel orientation and anchored to it by cross-links between its α chains and the telopeptide termini of type II, XI and other type IX collagen molecules. Since the globular domain and its adjacent helical domain are oriented away from the fibril it allows them to interact with other molecules in the perifibrillar space or potentially act as a spacer between individual fibrils composing the collagen fiber, a glue to bind the type II collagen lattice work or a device to facilitate fibril interaction with proteoglycans.

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1.5.4.2.3 Type XI collagen

Type XI collagen composes approximately 10% of the total collagen of articular cartilage. [53] It is an heterotrimeric molecule composed of 3 different α chains [(α_1-(XI)α_2(XI)α_3(XI)]. [51] with an α_3(IX) chain that is very similar to the type II collagen α_1(II) chain arguing that they may originate from the same gene. [62, 63] Most of the type XI collagen molecules are located within the collagen fibril and are covalently linked to type II collagen molecules by hydroxylysine-based aldehyde cross-links. They are believed to play a role in the regulation of fibril size by limiting its lateral growth. [64]

1.5.4.2.4 Type VI and X collagens

Type VI and type X collagen are the other two major types of collagen composing articular cartilage although they are not part of the collagen fibril itself. Type VI collagen is an heterotrimer composed of three different α chains [(α_1(VI)α_2(VI)α_3(VI)]. [51] It is mainly found in the pericellular region as a highly branched filamentous network based on the formation of tetramers using disulfide bonds. [65] It seems to be able to form associations with the small PG decorin and with hyaluronan [66, 67, 68] and is believed to serve as a link between the chondrocytes and the larger matrix fibrils. [51] Type X collagen is a homotrimer of the α_1(X) molecule. [51] It is characterized by a large non-helical globular domain present at its C-terminus. [69] In articular cartilage, it is found only near or around the hypertrophic chondrocytes of the calcified cartilage layer. [10, 70] and seems to play a role in the mineralization process of cartilage. [10]

1.5.4.2.5 Other Collagens

Type XII and type XIV collagens are the other types of collagen present in articular cartilage. They are also ubiquitous to other tissues and are found in cartilage only in very small quantities. They are both homotrimers and possess large globular domains at the end of the molecules. Their helical domain is also interrupted by short

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non helical segments. [53] They do not form fibrils but are often found closely associated with collagen fibers and have been termed fiber-associated collagens with interrupted triple helices or FACIT collagens. [53] Their role in articular cartilage has not been well documented.

1.5.4.3 Proteoglycans (PGs)

Proteoglycans are another component of the articular cartilage extracellular matrix. Most form a characteristic network between collagen fibrils in the interterritorial matrix [71, 72] contributing between 25% and 35% to the dry weight of cartilage. [49] They consist of a protein core to which are covalently attached many extended polysaccharide units called glycoaminoglycans. [73]

1.5.4.3.1 Glycoaminoglycans (GAGs)

Glycoaminoglycans are long unbranched polysaccharide chains made of repeating disaccharides that contain an amino sugar. There are two categories of GAGs: sulfated and non-sulfated. Only the sulfated GAGs are found in proteoglycans. [74] The five more common sulfated GAGs are chondroitin sulfate, keratin sulfate, dermatan sulfate, heparin sulfate and heparin of which only the first four are found in articular cartilage. Hyaluronic acid (HA) is also a GAG but differs from the others by the fact that it is not sulfated, [75] and that it does not require a protein to initiate its synthesis and therefore does not form proteoglycan molecules. It also differs from the other sulfated GAGs by its extreme length. A single HA molecule can possess as much as 10 000 disaccharide units giving it a molecular mass ranging from 3×10^2 to 2×10^3 kDa. In articular cartilage HA is associated with aggrecan through a link protein and with chondrocytes via CD44 cell surface receptor. [76]

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1.5.4.3.2 Aggregating proteoglycans

Aggregating proteoglycans are characterized by their ability to interact non-covalently with hyaluronic acid. The aggregating proteoglycan present in articular cartilage is aggrecan. [77, 78] It is composed of a core protein to which is covalently attached the GAGs keratan sulfate and chondroitin sulfate. Its presence in articular cartilage is intimately related to the ability of cartilage to withstand compressive loads. [78]

1.5.4.3.3 Aggrecan core protein

The aggrecan core protein consists of many different regions including some with specific characteristics allowing for the attachment of proteoglycans and interaction with HA. The amino-terminal region of the molecule possesses a series of disulfide-bonded domains that fold into 2 globular regions called G1 and G2. [79] The G1 region possesses 3 disulfide bonded domains, two of which conveys to the molecule the ability to interact with hyaluronic acid. The G2 region possesses two disulfide bonded domains called the proteoglycan tandem repeat. [80, 81] The carboxy-terminal region of the aggrecan protein also possesses a series of disulfide-bonded domains that correspond to a single globular region, G3 that has sequence similarity to epidermal growth factor, a lectin and a complement regulatory protein. The region between G2 and G3 is a long extended region where the GAG chains are attached [78] Over 100 consensus sites for attachment are present in the core protein. Those for keratan sulfate are closer to the G2 region whereas those for chondroitin sulfate are closer to the G3 region.

1.5.4.3.4 Proteoglycan aggregates

In the extracellular matrix of articular cartilage most of the aggrecan molecules are associated with hyaluronic acid to form proteoglycan aggregates. Up to 100 aggrecan molecules can be found bound to a single HA molecule via an ionic interaction

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through their G1 regions. [78] The HA- aggrecan interaction is usually stabilized by the intervention of a facilitator called link protein. [82, 83] which has significant sequence homology with the G1 region of aggrecan responsible for the interaction with HA [84-85] This interaction between aggrecan and the link protein seems to take place before their association with HA [86] but it is not yet clear whether or not all aggrecan / HA interactions are stabilized via the link protein as proteoglycan aggregates devoid of link protein have apparently been isolated [87]

1.5.4.3.5 Functional properties of proteoglycan

The large number of sulfated groups present on the GAGs gives the proteoglycan molecule a very high fixed negative charge density, which draws water into the tissue by osmosis. Because of the inextensible collagen network that surrounds the PGs, aggrecan is retained in a compressed form which creates a swelling pressure [88] giving cartilage its characteristic property to resist compressive load with minimal deformation, thereby supporting its function as a tough and resilient load-bearing surface.

1.5.4.4 Other matrix molecules

There are numerous other molecules present in the extracellular matrix of articular cellular. These include molecules such as biglycan, decorin, [89, 90] fibromodulin [91] perlican [92] thrombospodin [93] and cartilage oligomeric matrix protein [94] and many others. The roles of these non collagenous proteins have not been well elucidated but they appear to be involved in matrix organization, [95] mediating cell-matrix interactions [96, 97] and modulating chondrocyte phenotype. [95, 98]

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1.5.5 Articular cartilage physiology (chondrocyte metabolism)

Articular cartilage has two major functions; to provide a frictionless environment over which opposing joint surfaces can glide freely and to distribute loads evenly across the joint surface. Cartilage undergoes compression during joint movement and, is subjected to a wide range of mechanical loading forces which it needs to be able to withstand. The composition and the particular properties of the extracellular matrix give cartilage this ability. However extracellular matrix integrity is essential for the maintenance of these functions. Maintenance of cartilage homeostasis relies on the metabolic activities of chondrocytes which balance their anabolic and catabolic activities in order to adapt the ECM to the functional demands (mechanical loads) and various biologic stimuli (growth factors, cytokines, etc...). The anabolic activities of chondrocytes are characterized by the synthesis of the molecules composing the extracellular matrix and molecules preventing its degradation or favoring its synthesis. The catabolic activities are characterized by the synthesis of enzymes capable of degrading the matrix component i.e. mainly metalloproteinases.

Although it was believed for a long time that articular cartilage was an inert tissue, [99, 100, 101] we now know that when compensating for its low cell density, articular cartilage metabolism is almost equal to themetabolism of other tissues [102] with the difference that it is predominantly anaerobic (95%). [103, 13] The avascular characteristic of cartilage is responsible for this particularity creating a low pO_2 within the matrix which reduces its availability to the cells. Aerobic metabolism may take place under certain circumstances and uses the hexose monophosphate shunt pathways. [104, 105] The nutrients necessary for the chondrocytes metabolic activities reach the cells by diffusion from the synovial fluid through the matrix, probably assisted by the intermittent "pumping action" of weight bearing. [106] Secondary metabolites, such as

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lactic acid and CO_2, generated by glucose utilization are eliminated by the reverse route. [107]

1.5.5.1 Energy metabolism

In order to be able to carry out their distinctive activities, chondrocytes require energy. It is supplied to the cells by the conversion of carbohydrate to ATP (adenosine triphosphate) via anaerobic glycolysis that produces two ATP molecules and two lactic acid molecules per molecule of glucose. Glucose membrane transporters have been identified in chondrocytes. The family of GLUT transporter has been detected and GLUT3 is the most prevalent isoform in both human and bovine chondrocytes. [108] It is a carrier protein system that transports glucose into the cells by facilitated diffusion. [109] As mentioned above lactic acid is the most important byproduct of anaerobic glycolysis and it needs to be eliminated from the cell to maintain physiologic pH. Because lactic acid exist almost entirely as H^+ and lactate$^-$ ions at physiological pH the transport mechanism commonly involves a family of membrane-bound H^+ - lactate$^-$ cotransportor carrier proteins called monocarboxylate transporters (MCT). [110] The high capacity, low affinity MCT4 isoform has been identified as the major carrier in both human and bovine chondrocytes. [107]

1.5.5.2 Chondrocyte homeostasis

Chondrocytes are responsible for maintaining the integrity of the extracellular matrix. Therefore it is essential that they maintain their intracellular homeostasis to be able to perform their function. Chondrocytes exist in an unusual and variable ionic and osmotic environment. The high fixed negative charge of the extracellular matrix causes the cations (principally Na^+, K^+, Ca^{2+}) and proton concentration to be much higher than in serum while anions concentration remain relatively low. [111] The extracellular matrix is composed of approximately 80% water creating significant osmotic pressure.

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Furthermore, mechanical loading causes changes in the ionic and osmotic content of the matrix [112, 113] requiring adaptation response from chondrocytes. This response involves mechanisms necessary to maintain intracellular homeostasis but also changes in the synthesis of matrix components. [114, 115, 116, 117, 118] Ion transport mechanisms are involved in both of these processes.

The Na^+, K^+ - ATPase is responsible for the maintenance of the low Na^+:K^+ intracellular ratio and cell volume. This involves the active extrusion of Na^+ from the cell and the active entry of K^+. Studies in bovine and human cartilage have identified the presence of multiple isoform of the enzyme in chondrocytes. [119,120] One of them, the ß2 isoform is also an adhesion molecule [121] and could be responsible for transmitting information from the extracellular matrix to the intracellular environment. [122-123] The density of Na^+, K^+ - ATPase at the cell surface varies with cartilage depth and is more abundant in the middle zone which could be related to the higher compressive force seen in that area. [119, 124] The $Na^+/K^+/2Cl^-$ cotransporter is a source of sodium entry [125, 126] along with tetrodotoxin sensitive Na^+ channels, [127] the epithelial sodium channel, [128] the slow passive no specific Na^+ leakage [129] and the Na^+ X H^+ exchanger which is mostly involved in pH regulation. [130]

The chondrocyte extracellular environment is unusually acidic because of the large amount of protons attracted by the fixed negative charge of the extracellular matrix and, the lactic acid generated by the anaerobic metabolism of chondrocytes. Therefore intracellular pH regulation is critical to maintain normal cellular functions. In chondrocytes, contrary to most cells, pH regulation seems to involve a unique pathway involving the Na^+ X H^+ exchanger. [130] Two distinct isoforms have been identified in chondrocytes; NHE1 and NHE3. [130]Anion exchanger are present in chondrocytes [131] but they seem associated with the transport of sulphate anions into the cell [132]

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rather then to base extrusion. Proton extruding ATPases may be present but their existence is controversial. [133]

Intracellular Calcium (Ca^{2+}) levels need to be strictly regulated because of the role of Ca^{2+} as a potent second messenger capable of initiating a wide variety of cellular events. [134] In chondrocytes the extrusion of calcium is regulated by a Mg^{2+}/ATP/calmodulin dependent $Ca2^{+}$-ATPase which function against a high gradient. [135] The other transporter responsible for Ca^{2+} extrusion, the Na^{+} X Ca^{2+} exchanger has not been identified in chondrocytes so far. Mechanisms for calcium entry still need to be elucidated but recently there has been evidence of the presence of N-type voltage-sensitive calcium channel [136] and stretch activated calcium channels. [137] Stretch activated calcium channel may play an important role in mechanotransduction following mechanical loading.

1.5.5.3 Biologic response to mechanical loading

In order to be able to carry out its functions cartilage needs to be able to react and adapt to changes in the joint environment. Weight bearing and joint motion submit cartilage to a wide range of mechanical loads and forces. Numerous studies have demonstrated that cartilage is able to alter the composition of its extracellular matrix in response to mechanical loading. In mature joints, prolong limb immobilization leads to a loss in extracellular matrix proteoglycans and decrease in their aggregation [138, 139, 140, 141] while moderate exercise stimulate matrix synthesis. [142, 143, 144] In vitro studies using cartilage explants or chondrocytes seeded in agarose constructs show that chondrocytes respond to static compression by decreasing protein and proteoglycans synthesis [116, 117, 145]. On the other hand, dynamic compression at certain frequencies and amplitudes has been shown to increase synthesis of these matrix components. [117, 145] Furthermore the synthetic response to dynamic compression

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will vary with cartilage zone [146] and the degree of cartilage maturation. [147] The mechanisms involved in chondrocytes responses to mechanical stimuli are still poorly understood but they seem to be closely associated to various changes occurring in the ECM. It is known that deformation of the extracellular matrix caused by compression will produce mechanical, electrical and physicochemicals signals that may play essential roles in the modulating chondrocytes metabolic activities. [148-149] Loads applied to the articular surface cause cartilage deformation which may lead to changes in chondrocyte shape and, membrane stretching that could influence cellular metabolism. [150, 135] Deformation of the matrix causes fluid flow [117, 151], and movement of the counter-ions relative to the fixed charged groups on the proteoglycans of the matrix, altering charge density around the cells and creating streaming potential. The change in charge density in the matrix also affects extracellular pH, osmotic pressure and osmotic pressure gradients. [116, 117, 152] These are all potential signals indicating to the cell the need for matrix alterations. Another possible mediator could be the alteration in molecular transport of nutrients and metabolites to and from the chondrocytes induce by increased fluid flow. [116, 151, 153, 154] All of the above mechanisms could, individually or collectively, affect chondrocytes metabolic activities of and change the ECM composition. There is increasing evidence that one of those mechanotransduction pathways include direct interaction between the ECM components and the cell via the cell adhesion molecules intergrins. They appear to serve as mechanoreceptors that recognize the original mechanical stimulus and initiate a cascade of intracellular events resulting in the modulation of the expression of genes necessary to maintain cartilage homeostasis. [155, 156] The general mechanism seems to involve the binding of type II collagen or fibronectin to integrins which interact with elements of the cytoskeleton and focal adhesion complex proteins. Phosphorylation of these proteins seem to activate a

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cascade of events still not well characterized that leads to the activation of transcription factors regulating gene expression. [157-160]

1.5.5.4 Biologic response to growth factors, cytokines and other molecules

Numerous factors present in the joint environment have been identified as being able to affect synthesis of matrix components. Some of the most studied are probably the growth factors including molecules like insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMPs), that for most part stimulate synthesis of ECM components. Cytokines are another family of well studied molecules, most of them are known for their role in matrix degradation mainly through their inhibitory effects on the synthesis of matrix components and the stimulation of the production of degradative enzymes. The two most renown are probably interleukin-1 (IL-1) and tumor necrosis factor (TNF). Also present in the synovial fluid or stored in the ECM are a wide variety of other molecules (degradation products, inflammatory mediators such as prostaglandins and nitric oxide, free radicals, hormones and probably many other unidentified substances) that can affect chondrocyte metabolic activity. For the most part the effects of all these molecules on cartilage metabolism are beyond the scope of this dissertation. The effects of the factors directly implicated in the project will be reviewed in more details in a later section.

1.5.6 Articular Cartilage healing

1.5.6.1 Biology of articular cartilage healing

Damaged articular cartilage has very limited repair capacities and the mechanisms responsible for healing are inefficient at reforming a functional hyaline articular surface. [161-163] Partial thickness lesions in mature cartilage do not heal spontaneously. [164-166] Full thickness chondral lesions heal with repair tissue

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composed mostly of fibrocartilage that lacks the biochemical and biomechanical characteristics of normal hyaline articular cartilage, [167-169] leading to mechanical failure with prolonged use. [170]

There are three mechanisms by which full thickness chondral lesions heal; intrinsic repair, matrix flow, and extrinsic repair. Intrinsic repair relies on the limited capabilities of the remaining chondrocytes to proliferate and synthesize increased amounts of extracellular matrix. It is the least important mechanism of cartilage healing and also the least effective. [171] Matrix flow is described as the migration of lips of normal cartilage form the periphery of the lesion toward the center of the defect in an attempt to fill the defect. [171] It has been described as a relatively important part of the healing process in femoral defects but is almost nonexistent in lesions of the carpal bones. [168] Extrinsic healing is probably the most effective way cartilage has to repair itself. It is believed to rely on the migration of undifferentiated mesenchymal stem cells of the bone marrow into the cartilage lesion. Once in the defect, those cells proliferate and differentiated under the influence of growth factors also thought to come from the underlying bone marrow. The cells eventually start producing elements of the cartilage extracellular matrix to fill the defect. [171] Some reports suggest that mesenchymal cells migrating from the synovial membrane may also be implicated in that process. [172] All three healing mechanisms take place all at once during the healing of cartilage lesion but unfortunately they fail to regenerate a functional hyaline surface.

With the help of extended research done on spontaneous cartilage healing the sequence of events taking place in the aforementioned extrinsic healing process are starting to come to light. Rabbit models have dominated cartilage resurfacing research; therefore most of the descriptions of the healing process have been done in that species. [173-176] Most likely the sequence of events leading to the formation of repair tissue is the same between different species but the temporal component and the extent

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of each phase might be different from species to species. The same thing can probably be said for differences in healing seen in young and old subjects, [173, 174] in lesions situated in different location and in lesion of different depth and size, [176, 177, 168, 169]

Cartilage response to full thickness injury is similar to the response seen in other tissue. It is divided into three distinct phases: necrosis, inflammation and repair. [178] The necrotic phase begins immediately after the injury and is characterized by various degrees of cell death observed at the margins of the lesion. Cell death can be caused by mechanical trauma or by the induction of apoptosis. [179] Cell death may extend as far as 400 μm from the edge of the defect and be detected for as long as ten days after defect creation. [179] The inflammatory phase also starts immediately after wounding. Within a few hours of injury the lesion is filled with a blood clot that organizes into a fibrin network as quickly as one to two days after injury. [175, 180] Presence of inflammatory and undifferentiated mesenchymal stem cells soon follows. In rabbit, histologic evidence of their presence has been reported in the fibrin clot as soon as two days after the creation of experimental defects. [175] As cell migration takes place, organized invasion of the clot by capillaries of the bone marrow progresses which marks the start of the repair phase. Again in a rabbit model, 5 days into the repair process the defect is entirely filled by capillaries and undifferentiated mesenchymal cells. [175] It also appears that bone remodeling starts early as evidence of osteoblastic activity has been shown as early as three days after wounding. [175] In rabbits, by day 7 most of the fibrin clot has been removed and has been replaced by granulation tissue where undifferentiated mesenchymal stem cells are actively dividing. Healing in rabbit defects progresses rapidly, chondrogenesisis already occurring by day 10, as evident by the presence of matrix staining by safranin O. Cells with histologic characteristics of chondroblasts are present in the deeper layer of the repair tissue by day 12. For the

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next 2 weeks differentiation of the repair tissue continues. Differentiating mesenchymal cells produce a fibrocartilaginous tissue in the surface zone that contains flattened chondroblasts. In the zone between the surface and the bottom of the defect, marrow mesenchymal cells differentiate into rounded chondroblasts that synthesize a cartilage matrix composing most of the repair tissue. According to the authors of this study, by week four defects are filled by a well developed layer of typical articular cartilage containing chondrocytes. Changes seen in the following 6 to 8 weeks are associated to maturation of the repair tissue. [175] Throughout the healing process there was no evidence of healing coming from the lesion edges where the area of necrosis remained relatively inactive. Evidence of chondron formation was seen in the deep layers of the cartilage indicating some degree of cell proliferation. Integration of the repair tissue to the normal adjacent cartilage was delayed compared to the reformation of hyaline cartilage but did occur by week 24.

Models of cartilage healing in other species have not described the very early stage of repair but it is probably safe to assume that formation of the blood clot, fibrin network and migration of the mesenchymal cells and blood vessels occur in the same way if not in the same time constraint. However, the proliferation, differentiation and matrix synthesis stage may not happen to the same extent. In a horse model with medial condylar defects, part of the fibrin clot remained detectable in combination with granulation for up to two weeks after initial defect creation. [181] Eight weeks into the healing process granulation tissue was still present and hyaline-like tissue only constituted 12% of the repair tissue while fibrous tissue and fibrocartilage each composed approximatively 30% of the repair tissue. A long term study using that same equine model showed that even one year after defect creation, repair tissue only filled 52% of the defect. The repair tissue was composed mainly of fibrocartilage with hyaline-like tissue comprising 20% of the repair tissue. [182] Other studies performed on the

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horse using carpal defects as their model showed evidence of poorer healing in these locations. One study reports 39.3% filling after 21 weeks while another only 20.87% after 12 months. [183, 184] These studies also show a very low amount of hyaline cartilage in the repair tissue with percentages lower than 5%. [184, 185, 170]

The molecular events taking place in the early response to cartilage injury have not been studied extensively but a few studies have reported on the expression of matrix components during the early stage of healing. Two studies conducted in equine femoral defects reported type II collagen and aggrecan mRNA expression as early as 2 weeks after defect creation indicating early chondrogenesis. Messenger RNA signal increased with time for both proteins [181] but, according to one study, expression levels remained low even after 12 weeks, suggesting incomplete chondrogenesis. [186] During that same time message for type I collagen was also expressed but did not appear to change over the course of the project, another indicator of incomplete cell differentiation. [181] Pattern of mRNA expression showed that at 2 weeks, type I collagen was already seen in the deep layer and intermediate layer of the repair tissue and had progressed to all layers by week 4. Aggrecan and type II collagen mRNA were first detected *in situ* at 4 weeks. Aggrecan was present in both the deep and intermediate layers while type II collagen was only seen in the deep layer. By 6 weeks both signal were detected in all layers. Protein expression paralleled mRNA expression but with a lag phase. By week 8 protein expression for both aggrecan were seen in all layers but were predominant in the deep and intermediate layers. Type I procollagen was still being expressed but at a lower intensity. These findings confirm what has been shown by histologic studies; the repair process starts at the base of the defect and gradually progresses to the surface.

Growth factor expression is probably a key factor in modulating cell migration, proliferation and differentiation during that early healing period. An enormous amount of literature has been published on the effects of various growth factors on chondrocyte

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proliferation, differentiation and capacity of producing matrix components. There are a growing numbers of studies reporting up regulation of growth factor expression and down regulation of their receptors in disease like osteoarthritis where chronic hyaline cartilage damage is present [187, 188, 189] but, still very few reports of the effects of acute articular damage and early healing on growth factor expression. One study performed in horses looked at IGF-1 mRNA expression using RT-PCR and found that an increase in its expression was not seen until 4 weeks after defect creation. This was followed by a gradual increase up to week 8 and a significant decrease on week 16. [186] A second study evaluating the immunochemical expression of growth factors in cartilage defects in rabbit ears showed minimal staining for TGFβ_1 and TGFβ_3 at the wound edges the day following surgery. [190] Positive staining for those two proteins increased from day one to day 3 but remained located around the margins of the defect. TGFβ_1 expression remained relatively the same until day 14. Staining intensity for IGF-1, IGF-II and FGF-2 increased from day 3 to day 7 when they reached peak levels along with TGF-β_2. Staining for all growth factors had returned to baseline levels by day 28. The increase in growth factor expression correlated with proteoglycans depletion of the wound edges and their decrease with the restoration of proteoglycan content. [190] These results suggest that cartilage response to acute injury does involve the up regulation of the expression of growth factors and that it seems dependent on the matrix depletion of proteoglycans after wounding. However, this model is different from the full thickness chondral defect model where the cells from the bone marrow would probably be the most important source of growth factors.

1.5.6.2 Inadequacy of articular cartilage repair

Research has firmly established that full thickness cartilage lesions heal themselves as long as access to the underlying bone marrow is provided. It has also

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been demonstrated that the healing process is in general inadequate and does not reform tissue with the structural and biomechanical characteristics of hyaline cartilage leading to tissue failure overtime. Numerous factors have been implicated in this incapacity to generate tissue with long term functionality. The cartilage own structure and metabolism may be in the forefront of the problem. Mature adult cartilage is a hypocellular tissue and cells are isolated in a dense extracellular matrix making cell migration and proliferation difficult. Low proliferation rate may also be explained by cartilage low cell turnover. [191, 192] Furthermore, cell isolation makes communication between cells more complicated; making them rely on paracrine pathways rather then direct cell contact to exchange information. [179] Chondrocytes from cartilage of mature adults have a decreased capacity to synthesize matrix components in response to injury, making intrinsic repair almost inexistent. [174] The avascular character of cartilage may also come into play. It deprives the injured area of inflammatory cells and blood born factors that play important parts in the response to wounding in other tissue. [191, 173] Adults also have a decreased population of mesenchymal stem cells capable of migrating in the lesion following injury decreasing the capacity of extrinsic repair. [174]

Lack of repair tissue integration to the adjacent cartilage and underlying subchondral bone has also been a characteristic of inadequate cartilage repair. [193, 194] Poor attachment to the subchondral bone seems to be closely related to an incomplete removal of the calcified cartilage layer. It has been suggested that the presence of the calcified cartilage layer may impede the bonding of the repair tissue to the base of the defect by preventing the incorporation and anchoring of the newly synthesized collagen fiber to the remodeling subchondral bone. [195] Poor lateral integration to the edges of the defect has been associated to the anti-adhesive properties of some of the component of the extracellular matrix. Decorin, aggrecan and

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biglycan can inhibit cell adhesion to macromolecules such as fibronectin and collagen, therefore interfering with integration mechanisms. [194]

1.5.7 Growth factors and cartilage healing

Growth factors are soluble molecules involved in intercellular communications. They produce their biologic effects mostly in a paracrine or autocrine manner, through the interaction with high affinity cell surface receptors. This interaction initiates a cascade of molecular mechanisms affecting the expression of specific genes and therefore protein synthesis leading to a change in the cell activity pattern. So far, the major growth factors shown to be involved in cartilage healing are insulin-like growth factor-1 (IGF-1), [196-198] transforming growth factor beta (TGFβ), [196, 199, 200] fibroblast growth factor (FGF), [201-203] and bone morphogenetic protein-2 (BMP-2). [204-206] Others have also been implicated but have not been studied as extensively. Among them are insulin, [207] platelet derived growth factor (PDGF), [208] and epithelial growth factor (EGF). [202] Each one of these growth factors plays a specific role in cartilage healing and most of them affect more than one stage of the healing process.

1.5.7.1 Insulin like growth factor-1 (IGF-1)

Probably the first times IGF-1 was reported to have an anabolic role in cartilage was in 1957 by Salmon and Daughaday. They described the factor as a growth hormone-dependent serum factor able to stimulate ^{35}S-sulphate incorporation into cartilage in vitro. [209]

The human IGF-1 gene consists of 6 exons located on chromosome 12. The equine gene possesses 5 exons. Both code for a large mRNA precursor that is alternatively spliced to yield two translated pre-pro-peptides that will then be cleaved into the IGF-1 protein. [210, 211] Human IGF-1 is a single polypeptide with a molecular mass of 7.5 kDa and with significant sequence homology with insulin. [212-214] The

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equine IGF-1 has an amino acid sequence identical to its human counterpart. [210] IGF-1 was originally thought to be produced by the liver and act like an hormone but, it is now well known that it is produced in a wide type of cells and tissues, suggesting that it acts via autocrine/paracrine as well as endocrine pathways. [215-217]

IGF-1 mediates his biologic effects through its interaction with its specific cell surface receptors. So far two IGF-1 receptors have been identified; each one with its own structure and binding specificity. The type I IGF-1 receptor is thought to mediate most of the known effects of IGF-1. [218] The precise role of the type II receptor is still not well understood but it as been suggested that it may serve as a reservoir and/or clearance mechanism for IGF-2. [219] Regulation of the IGF-1 effects on its target cells is partly governed by IGF-1 binding proteins (IGFBPs). These extracellular proteins modify the interaction of IGF-1 with its receptors. [220] To date seven IGFBPs have been identified, cloned and sequenced. The amino acid sequence homology between each of them is relatively high but, they each have a distinct structure and biochemical properties and, exhibit tissue specific expression. IGFBPs 2 -5 have been identified in joint tissues and IGFBP-3 and 4 appear to be of major importance in these tissues. [221-222] IGFBPs are present in the cartilage territorial matrix bound to fibronectin and forming a complex with IGF-1, indicating that in mature cartilage IGF-1 may be stored in that fashion. These IGF-1 stores may help maintain a relatively constant level of IGF-1 and the local increase in matrix synthesis following cartilage damage may be the result of the release of IGF-1 from those stores. [223]

IGF-1 has been shown to play a pivotal role in cartilage anabolic metabolism as well as regulation of growth and differentiation of articular cartilage. [197] Its effects have been evaluated in various experimental *in vitro* systems using different species. These models include cell culture of fetal and adult chondrocytes, cultured cartilage explants and repair tissue as well as immortalized cell lines. Equine cartilage has been

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the focus of a number of studies evaluating the effect of IGF-1 on that tissue metabolism. The results obtained were similar to those of other studies where other species or human tissues were used. In a study by Schofield *et al.*, using both fetal and neonatal chondrocyte in culture, physiologic concentrations of IGF-1 showed mitogenic properties as demonstrated by an increase in the number of cells in S phase and an increase in DNA ^{3}H thymidine incorporation. [224] Another study performed by Nixon *et al.* on monolayer culture of fetal chondrocytes confirmed the proliferative effects of IGF-1 on fetal cells and, demonstrated an increase in proteoglycan production with a greater proportion of larger proteoglycan monomer. [225] Two other studies performed by the same group using various concentration of IGF-1 on chondrocyte-fibrin cultures showed similar results. Furthermore it demonstrated that IGF-1 was also capable of increasing type II collagen synthesis by chondrocytes and that the effects of IGF-1 on cartilage were concentration dependent. It also showed that supraphysiologic concentration did not seem to have detrimental effects on the cells and they were able to maintain their phenotype in culture. [226-227] Some of the effects of IGF-1 on equine cartilage appear to be age dependent. Bayliss and Platt demonstrated that although proteoglycan synthesis was stimulated in young and adult cartilage explants higher concentration of IGF-1 were required for maximal stimulation of adult cartilage. However, age had no effect on the size of proteoglycan produced after stimulation with IGF-1. Furthermore, IGF-1 was able to reduce the rate of turnover of newly synthesized and endogenous proteoglycans in explants of all ages. [228] IGF-1 also has anti-catabolic activities and is able to reverse some of the effects of IL-1 on articular cartilage. A study using equine cartilage explants showed that the addition to IGF-1 to IL-1 conditioned explants was able to abolish the increase in degradation rate of proteoglycans induce by IL-1, reestablish proteoglycan synthesis decreased by IL-1, and restore proliferation rate similar to the one seen in controls. [229] IGF-1 also seems implicated in mesenchymal

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stem cells differentiation into chondrocytes. Isolated bone marrow mesenchymal stem cells placed in presence of IGF-1 for 13 days showed significant chondrogenic transformation as demonstrated by markers of chondrocytic function such as phenotypic condensation, collagen type II mRNA and protein production and, proteoglycan accumulation. [230]

The expression of IGF-1 is modulated by a wide variety of factors that are also implicated in cartilage homeostasis. Growth factors such as basic fibroblast growth factor (bFGF) and growth hormone were shown to stimulate the secretion of IGF-1. [231, 232] Growth hormone seems to work through both direct and indirect pathways to stimulate IGF-1 mRNA production in normal cartilage while it does not seem have any effect on osteoarthritic chondrocytes. [233-235] Furthermore IGF-1 expression is also regulated through a positive autoinductive autocrine/paracrine loop where a physiologic concentration of IGF-1 will induce the expression of IGF-1 mRNA within 1 or 2 days. [236] Other molecules also have stimulatory effect on IGF-1 expression, PGE_2 being one of those. It has been suggested that PGE_2 may act as a secretagogue of IGF-1 and that the latter growth factor may mediate, via an autocrine loop and the IGF-1 receptor, some of the anabolic effects of the ecosanoid on cartilage metabolism. [237] While some molecules and growth factors have some stimulatory effects on IGF-1 expression some others have been shown to decrease its expression. Transforming growth factor-β inhibited the release of IGF-1 in cultured chondrocytes and IL-1 was able to downregulate IGF-1 mRNA levels and induce the secretion of IGF-1 receptors and IGFBPs, reaffirming that an autocrine loop regulates IGF-1 functions. Tumor necrosis factor α and interleukin-6 had similar effects. [231, 238-240]

As discussed in the previous paragraphs, IGF-1 plays an important role in cartilage metabolism. It even has been mentioned as the most important growth factor regulating chondrocyte proteoglycan metabolism. [241] In spite of this, very few studies

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have looked at its potential to enhance healing in chondral defects. In an equine model of spontaneous repair, IGF-1 mRNA has been show to be expressed from week 2 following defect creation until week 16. [186] A study performed in an equine model of full-thickness chondral defect showed that the addition of IGF-1 to fibrin composites implanted in defects was able to enhance healing by increasing histologic healing scores, type II collagen content and attachment of the repair. [242] Another study performed by the same group but this time using IGF-1 in a cell-based implant also showed enhanced healing. In this model a beneficial effect IGF-1 was demonstrated by enhanced chondrogenesis in the cartilage defects, including incorporation into surrounding cartilage. Gross filling of the defects was also improved, as well as the repair tissue type II collagen content and the proportion of cells producing type II collagen. [243] A recent study using genetically modified mesenchymal stem cells over-expressing the IGF-1 gene in partial thickness defect in rat femurs showed similar results. The authors describe the production of repair cartilage of hyaline morphology containing a type II collagen-positive but type I collagen-negative proteoglycan-rich matrix that restored the articular surface while uninfected cells failed to fill the defect or produced fibrous tissue expressing mainly type I collagen. [244] However, another group using the rabbit as a model showed no effect of IGF-1 on cell replication, chondrocyte proteoglycan synthesis or defect filling. [230]

1.5.7.2 Other growth factors

Transforming growth factor-β (TGFβ) is a pleotropic growth factor as its effects vary depending on the concentration used and the composition of the micro-environment. [198] Like IGF-1, its effects have been studied in a wide variety of experimental systems. It plays a role in the regulation of chondrocytes mediated matrix turnover by affecting type II collagen and proteoglycans synthesis. [196, 198, 206] It

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can enhance proliferation of chondroprogenitor cells, [198, 206] and promote chondrogenic differentiation of mesenchymal stem cells while inhibiting osteogenesis and adipogenesis. [245-248] In other cases, it has been shown to confer a distorted phenotype to chondrogenic cells [205] and stimulate fibroblastic differentiation of cells from repair tissue. [196] It also has been demonstrated that TGFβ has chemotactic activities on mesenchymal stem cells of different origin. [203, 205] Basic fibroblast growth factor (bFGF) has the capacity to positively affect matrix composition, [196] is a potent mitogen, [203, 205] and its chemotactic properties approximate those of TGFβ. [202, 194] Like IGF-1, it has been used in a chondral defect model and has been shown to enhance cartilage healing by promoting cell differentiation and matrix component synthesis. [201] Although the role of bone morphogenetic proteins (BMPs) in bone healing has been studied extensively, their role in cartilage healing and chondrogenesis is not well defined but, as in fractures, their primary responsibility seems to be associated with cellular differentiation. BMP-2 appears to be necessary for the chondroprogenitor cell's early differentiation phase [204] and last phase differentiation into hypertrophic chondrocytes. [205] In a rat model of partial chondral defect it has shown positive effect on cartilage healing but was associated with the formation of osteophytes. [245]

1.5.8 Anti-inflammatory molecules and cartilage healing

The use of anti-inflammatory drugs and/or chondroprotective agents following arthroscopic debridement of full thickness chondral lesions in the horse is part of the routine post surgical management of a case, along with a period of stall rest and controlled exercise. Control of the inflammatory reaction following surgery has always been an important part of the treatment of chondral defects and, even though few studies have directly looked at the effects of these drugs on the mechanisms of cartilage

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healing, a lot is known about their effect on cartilage in general. The effects of corticosteroids on cartilage healing have been studied and, overall they seem to have a detrimental effect on the quantity as well as on the quality of the repair tissue. [249, 250] In one equine study of full thickness osteochondral defects the use of sodium hyaluronate and polysulphated glycosaminoglycans did not affect the biochemical composition of the repair tissue. [251] However in *in vitro* and *in vivo* models of acute loss of cartilage proteoglycans induced by IL-1 or enzymatic digestion, hyaluronic acid was able to restore cartilage proteoglycan content. [252, 253] The effects of non steroidal anti-inflammatory drugs (NSAIDS) on cartilage can be debated. Effects will vary depending on the drug being studied and its concentration. Ibuprofen was shown to have a detrimental effect on the healing of bone and cartilage in the temporomandibular joints of rabbits. [254] In one equine model phenylbutazone was shown to decrease proteoglycan synthesis, [255] while in another it reduced the lost of proteoglycan from cartilage explants and decreased proteoglycan synthesis only at high doses. [256]

1.5.8.1 Interleukin-1 receptor antagonist (IL-1ra)

The degree of inflammation in a joint following surgical treatment of articular cartilage lesions will vary from case to case depending on the extent and the chronicity of the damage. Nevertheless some degree of inflammatory reaction will be present if only related to the surgical procedure. Work at the molecular level and with in vitro models has allowed for a substantial advancement in the knowledge of the pathophysiology of joint disease. Valuable information has been gained on the molecular events affecting cartilage destruction and cartilage healing. Loss of cartilage homeostasis with imbalance in favor of the catabolic pathways typically results in cartilage matrix degradation and often leads to clinical manifestation of joint disease.

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Inflammation plays a pivotal role in the initiation of the catabolic events. [257] Inflammatory cytokines such as interleukin-1 (IL-1) are responsible for the majority of the cellular events leading to matrix degradation and loss of articular cartilage. [258, 259] Blocking the inflammatory effects of IL-1 in the hope of providing a more normal joint environment and thus promoting cartilage healing would probably be beneficial.

Interleukin-1 receptor antagonist is a natural inhibitor of IL-1. The molecule was first recognized as a 22-25 kDa IL-1 inhibitory bioactivity in the supernatant of monocytes cultured on adherent IgG [260] and in urine of patients with myelomonocytic leukemia. [261, 262] The human IL-1ra gene has 4 exons and is located on chromosome 2 along with the genes of IL-1α, IL-1β and type I and type II IL-1 receptors. [263, 264] The mRNA codes for a protein of 152 residues that can be glycosylated. [265] There are also three intracellular forms of IL-1ra but their functions have not been well characterized but suggest the presence of a more complex IL-1 regulating system. [266, 267] The inhibitory effects of IL-1ra are mediated by the binding of the molecule to the IL-1 cell surface receptors. IL-1ra is a pure antagonist as it has no known or detectable agonist effects. Expression of IL-1ra can be induced by a variety of cytokines in a wide range of cell types. IL-1α, IL-1β, IL-3, IL-4, IL-10, GM-CSF, and TNF have all been shown to increase the expression of IL-1ra. [265, 268]

IL-1ra has been shown to be present in the synovial fluid and synovial tissue of human patients with rheumatoid arthritis and osteoarthritis. [269, 270] It has been reported that the levels of IL-1ra were elevated in the synovial fluid of 80% of the patients with rheumatoid arthritis and 30% of the patients with infectious or inflammatory, non-rheumatoid arthropathies. [265] According to studies in different animal models of inflammatory arthritis, a deficiency of IL-1Ra relative to IL-1 leads to more severe disease and even to the spontaneous development of arthritis as observed in IL-1Ra

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knockout mice. [265] Cultured articular chondrocytes appear to constitutively express IL-1ra. [271]

The potential beneficial effects of IL-1ra in an *in vivo* model of healing chondral defect have not been investigated but, the anti-catabolic and anti-inflammatory effects of the molecules on tissue of the joint have been well studied and the results suggest that it should have similar effect in cartilage healing. IL-1ra has shown the ability to inhibit prostaglandin production by chondrocytes and synovial cells, and collagenase production by IL-1 activated synovial cells. [272] IL-1ra has also been show to suppress IL-1 activated metalloproteinase production by chondrocytes. [273] In an experimental model of arthritis, Il-1ra was able to block IL-1 induced inhibition of proteoglycan synthesis and monocyte infiltration into synovial tissue. [274] In a clinical trial with people with rheumatoid arthritis, a daily subcutaneous injection of IL-1ra significantly improved the parameters of disease when compared to placebo treated patients. Additionally a significant reduction in radiographic progression of joint disease was noted in the IL-1ra treated group. [275] The possibility to use IL-1ra in gene therapy for the treatment of arthritis has also been investigated. The first published study was able to demonstrate that IL-1ra delivered to the joints by an ex vivo technique using a retrovirus decreased the white blood cell count in the synovial fluid of rabbit joints injected with IL-1β. [276] The same group, using the same model also demonstrated transgene expression of IL-1ra inhibited synovial membrane thickening and hypercellularity as well as articular cartilage proteoglycan loss. [277] In a rabbit model of antigen-induced arthritis the use of the same viral vector and ex vivo technique showed that IL-1ra again, was able to decrease synovial fluid leukocyte infiltration and matrix catabolism but had no effect on other parameters of inflammation such as joint circumference. [278] A study in dogs using a cruciate model of osteoarthritis and a retroviral vector showed marked reduction of macroscopic and histologic lesions in the tibial plateaus and femoral

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condyles of cruciate deficient dogs expressing IL-1ra. In that particular model IL-1ra levels in synovial fluid was still detectable 4 weeks post treatment. [279] Direct delivery of IL-1ra to the joint using adenoviral vectors has also been studied. [280, 281] In an equine model of osteoarthritis, injection of an adenoviral vector carrying the equine gene of IL-1ra showed intra-articular expression of IL-1ra protein in the synovial fluid for a period of 28 days, resulting in significant improvement in clinical parameters of pain and disease activity, preservation of articular cartilage, and beneficial effects on the histological parameters of synovial membrane and articular cartilage. 280]

It was originally thought that large excesses of IL-1ra (up to 100 fold) were necessary to significantly inhibit the catabolic effect of IL-1. [265] However the more recent studies using frequent subcutaneous injection of IL-1ra or gene transfer of IL-1ra seem to show that a large molar excess of IL-1ra may not be necessary to reduce or inhibit pathologic parameters of joint disease. These results suggest that a low but sustained expression may also be beneficial.

1.5.9 Joint resurfacing techniques

Severe cases of osteoarthritis are still being treated with prosthetic joint replacement. However, this technique has significant limitations; synthetic surfaces do not duplicate the mechanical properties and durability of articular cartilage. [52] Therefore, with use, synthetic articular surfaces wear off and prostheses loosen requiring replacement. For this reason, numerous methods have been developed to improve cartilage healing in an attempt to regain long term use of the injured joint. Most biological resurfacing techniques are based on the assumption that increasing the number of cells present in the defect will promote and enhance cartilage healing. [282] This can be achieved in two different basic ways: stimulating repair by accessing endogenous cell populations present in the underlying bone marrow or by providing the

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defect with an exogenous cell source to promote healing. [283, 284] Many techniques rely on the penetration of the subchondral bone plate for a source of local undifferentiated mesenchymal stem cells. [283] The most popular techniques are spongialization and subchondral bone drilling, each one coming with its own limitations. Spongialization advocates the removal of sclerotic subchondral bone from the base of a full thickness defect. [285] However this technique compromises the biomechanical stability of the subchondral bone plate resulting in abnormal stresses that disrupt the new repair tissue. [184] Subchondral bone drilling allows access to the cancellous bone but the use of drills can cause significant thermal necrosis, limiting the amount of healing. [182] Techniques using exogenous tissue to provide a source of pluripotent cells have also been investigated. These include the use of allo- or autografts of various type of tissues (cartilage, [184] periosteum, [286] and osteochondral tissue. [287, 288]) Low chondrocyte viability and delayed immune response to allografts are problems that may contribute to long term failure. [289, 290] Techniques using autografts seem to have been more successful but show a tendency toward ossification of the repair tissue [291] and disappointing results when studies were extended and the models were put to the test of time. [184, 287, 292] These techniques also increase patient morbidity because of the need for additional surgery sites to harvest the grafts.

The latest techniques involve the implantation of various types of cultured cells into chondral defects. Autologous chondrocytes delivery systems have been used clinically and a two to nine year retrospective study shows good to excellent clinical results and good histological results in small focal lesions but when more extensive damage was present, when there were multiple lesions or when they were located on the patella the success rate dropped significantly. [293] Mesenchymal undifferentiated stem cells from the bone marrow have also been studied because of their potential to repair both subchondral bone and articular cartilage and, because they can easily be

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harvested in significant amounts. [294-298] Results in experimental models using either cells alone or cell seeded within various carrier matrices are promising. They show evidence of viable chondrogenic cells in the defects, staining with safranin-O and excellent bonding between the regenerated and adjacent normal tissue in one study. [296] All together these cell based implant techniques show encouraging results but, they have their drawbacks. They require more than one surgical procedure because of the necessity to harvest progenitor cells and necessitate prolonged period of cell culture before implantation is possible, increasing the chance of in vitro contamination and cell death, therefore increasing the risk of failure, delaying repair of the lesion and possibly increasing the severity of disease. Furthermore, experience and meticulous technique are required for a successful surgical implantation. [291]

Looking at the body of work that has been generated in the field of cartilage resurfacing, it becomes obvious that none of the described techniques has been entirely successful in generating adequate repair tissue. The most encouraging results were seen when an actively dividing population of cells that can differentiate into chondrocytes were used or accessed. What remains to be determined is the most effective method to access or implant those cells in the defect and how to persuade them to undergo the morphogenic process that will produce true articular cartilage. The research project presented here utilized a technique called subchondral bone plate microfracture to access the mesenchymal stem cells of the bone marrow.

1.5.9.1 Subchondral bone plate microfracture

The subchondral bone plate microfracture (SBPM) was first described by J. Richard Steadman who created the technique in the early 80's. [299, 300] SBPM is an arthroscopic technique that involves the use of an arthroscopic shaver to perform a rough debridement of full thickness chondral lesions, followed by the use of hand-held

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curettes for more precise debridement and removal of the calcified cartilage layer without affecting the subchondral bone plate. Custom made surgical bone awls are then used to perforate the subchondral bone plate at a 90° angle, to a depth of 2 to 4 mm every 3 to 4 mm. Adequate depth is evident when fat droplets and blood start pouring out from the microfracture holes. [301]

The perforation of the subchondral bone plate by the surgical awl access the components of the bone marrow and allows cartilage to react to injuries in a way more related to other tissues of the organism i.e. undergoes the three distinct phases of healing: necrosis, inflammation and repair. [302] Injured cartilage undergoes necrosis like any other tissue but in order for the healing process to progress to the inflammatory and repair phases it needs access to blood vessels, cytokines, growth factors as well as undifferentiated mesenchymal cells. [303] This access can be provided by the SBPM technique with some advantages over other techniques used in the past.

The SMBP technique produces microfractures with a spiculated and irregular rim of bone; this rough subchondral bone surface may favor better adhesion of the early blood and fibrin clot, [301] compared to mechanical drilling that leaves a smooth, polished surface less suited to clot adhesion. Furthermore, mechanical drilling has the potential to exacerbate cartilage necrosis and cause thermal bone necrosis because of excessive heat generation. [304] Other advantages of the SBPM include its simplicity and its relative inexpensiveness; any experienced arthroscopist can perform the procedure with little training and no additional cost but the price of the surgical awls. There is low patient morbidity because it is performed via arthroscopy. It takes advantage of the endogenous cell population for healing, eliminating the risk of immune reactions to transplanted tissue or the need for additional surgical procedures to harvest and implant grafts or cells. But maybe even more important, it does not jeopardize the ability to perform future procedures should they become necessary.

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A long term clinical study (average follow-up of 6 years on 100 patients) on the use of SBPM for the repair of full-thickness traumatic chondral defects has shown a significant improvement in all functional parameters studied as well as in the ability to walk 2 miles, descend stairs, perform activities of daily living, strenuous work and strenous sports. Eighty-six percent of the patients rated their knee as feeling normal or nearly normal after surgery. [303] The same study also looked at the effect of SBPM on 80 patients > 50 years of age with osteoarthrosis of the knee. The analysis showed, as for the first subjects of the study, a significant improvement in subjective complaints of pain and swelling, an improvement in walking 2 miles, performing strenuous work, sports and activities of daily living. Failure were associated to chronic lesions, severe narrowing of the joint space and axial misalignment. [303] The same group performed a similar study done on 266 high level performing athletes. [305] Improvement was noted in all the parameters studied and 71% of the athletes felt that they were able to return to competition at a level equal or superior to the one pre-injury.

It is important to note that the previous studies were not performed using a control group and precludes the comparison of the clinical outcome to non-treated control patients. It remains that those studies provide important information on factors affecting the clinical outcome of patients treated with the SBPM. Factors that seem positively correlated to better outcome are: removal of the calcified cartilage layer without disruption of the subchondral bone plate and early postoperative continuous passive motion with strict protected weight bearing. Factors that seem to be associated with failure or, less then satisfactory results are: abnormal mechanical axes, age, location of lesions, greater interval between injury and treatment and no or shorter physical therapy following surgery. [303, 305]

Although the SBPM has been used to treat full thickness chondral injuries in human patients for more than 20 years, few studies have evaluated the beneficial effects

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of the technique using a well characterized model in order to obtain an objective evaluation of the quality of the repair tissue. The research group headed by Dr. CW. McIlwraith, with the collaboration of Dr. R Steadman, was the first to report the effect of SBPM on healing of large chondral defects created in horses. [182] Full thickness chondral defects with removal of the calcified cartilage layer created on the proximal axial surface of the weight-bearing portion of the radial carpal bone and the medial femoral condyle were evaluated. Randomly selected carpus and the contralateral stifle were treated with SBPM and opposite joints were used as controls. The horses were submitted to a controlled exercised program and defects evaluated 4 or12 months post-operatively. Macroscopic evaluation showed that all defect sites were still apparent 12 months after creation. They were filled to various degrees with a white, glistening and irregular repair tissue that was firm to palpation and for the most part well attached to the surrounding tissue and subchondral bone. Subjective evaluation showed that tissue present in the treated defects seem more uniform and appeared to fill a greater percentage of the defect (74 % and 45% respectively) than the one in the non-treated group but, independently of treatment, there was no difference between the amount of the repair tissue seen in the defects at 4 and at 12 months. Histomorphometric analysis confirmed the previous finding with slightly lower percentages; 60% filling in treated defects and 39% in control defects. Even though the volume of repair tissue was increased in the treated defects, the histologic composition of the tissue with respect to percentage of fibrous tissue, fibrocartilage and hyaline cartilage was not statistically different over time or between joints or between treated versus control defects. However biochemical analyses were able to demonstrate that there was significantly more type II collagen in the repair tissue of the treated defects at 4 months for stifles and at 12 months for carpi. Microscopic analysis showed that tissue filling the microfracture sites was intimately associated with the rest of the repair tissue giving an overall better

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attachment of the repair tissue to the subchondral bone. It was also noted that in areas where calcified cartilage had not been removed the attachment of the repair tissue to the subchondral bone did not appear as good as when it had been removed. Overall, this first controlled study on SBPM was able to clearly show beneficial effects of the technique on healing of chondral defects; however in this study SBPM was incapable of altering the composition of the repair tissue and increase the percentage of hyaline-like cartilage.

In order to understand the early cellular and molecular events leading to an increase in the repair tissue type II collagen content, the same group performed a second study looking at the healing of femoral condylar defects over a period of eight weeks. [181] Although the study failed to show differences in location and temporal expression of the key components of the extracellular matrix, it showed an increased of type II collagen mRNA expression in the MXFX defects when compared to the non-microfractured one and it did give a good insight into the events taking place during early cartilage healing. A fibrin clot first fills the defect followed by the formation of granulation tissue, by week 4, fibrous tissue is already apparent and fibrocartilage could be seen 6 weeks following defect creation. Tissue with characteristics of hyaline-like cartilage does not appear in the repair tissue before week 8. RT-PCR detected the message for type II collagen and aggrecan as early as 2 weeks post defect creation with a gradual increased in expression over time. On the other hand, type I collagen mRNA expression remained relatively constant throughout that period. From the pattern of mRNA expression in the repair tissue it was demonstrated that the chondrogenesis originates from the bottom of the defect and then gradually extends upward toward the surface. Protein synthesis trailed mRNA synthesis but was expressed in similar patterns. By week 8, type II collagen was detected in the deeper layers of the repair tissue while aggrecan was spread more uniformly over all zones, indicating that

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proteoglycans production may precede type II collagen in the sequence of event leading to the formation of the repair tissue.

A second group headed by Dr M. Spector also studied the effects of SBPM. [306] Their study compared the healing of defects treated with a collagen implant seeded with autologous chondrocytes , SBPM alone or, combined with the collagen implant. They created defects in the trochlear groove of hound dogs and evaluated defect healing 2 hours and 15 weeks post-operatively. They used an arthrotomy technique, did not make an effort to remove the calcified cartilage layer and immobilized the joint for 10 days following surgery. Their results showed that 2 hours after surgery the defects were filled with blood clots, with better filling in defects implanted with the collagen matrix. Fifteen weeks postoperatively defects were filled with fibrocartilaginous and fibrous tissue with virtually no hyaline cartilage. The amount of repair tissue and its characteristics were the same regardless of group. However, when compared with untreated defects of control dogs from previous studies performed by the same group, [307, 308] SBPM treated defects were found to contain significantly more repair tissue but less hyaline cartilage. Also, calcified cartilage and subchondral bone remodeling was more frequent and extensive in the SBPM treated defects when compared to the controls. Although this study did not offer a direct comparison of treated and untreated defects and that the calcified cartilage layer was not removed, the results are similar to the one reported by McIlwraith et al. [182]

1.5.10 Gene therapy

Gene therapy is an experimental medical intervention that involves modifying the genetic material of living cells to fight disease. The procedure is defined by the delivery of functional genes into target cells to correct the effects of an abnormal gene, introduce a new function to a cell or in our case amplify the effect of an existing gene.

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There are two general methods used in gene transfer, direct *in vivo* and indirect *ex vivo*. The *in vivo* methods involves the injection of the vector directly into the target tissue, whereas the *ex vivo* method relies on cells genetically modifying in culture re-implanted at the disease site. Each one of these methods has its own advantages and disadvantages. In vivo delivery implicates the injection of viral particles and the production of viral proteins into the host which invariably results in some inflammation and immune mediated destruction of the transformed cells, therefore limiting the length of expression of the transgene. Furthermore since the vector is directly injected into the patient, selection of transduced cell is impossible, control over target cell population is more difficult and testing for the presence of recombinant virus is also impossible. Despite its disadvantages this method possesses numerous advantages that make it an interesting choice for treatment of joint disease and cartilage healing. The technique is simple and requires less invasive manipulation than ex vivo gene transfer since it can be accomplished by a single injection into the joint. This compared to the need to harvest cells, culture and transduced them in vitro before re-implanting them into the patient. These manipulations are time consuming and require the need for special facilities increasing the cost of the procedure and retarding treatment of the patient. On the other hand, it makes the selection of the population of cell to be transduced possible; it allows the re-implantation of cells known to be producing the transgene into the patient and avoids the introduction of viral particles into the diseased area.

The ability to transfer and appropriately express the genes encoding for the protein of interest is dependent on the availability of effective gene transfer vectors. Numerous vector systems have been used to transfer and express genes in joints. These vectors can be divided into two categories, the viral and non-viral vectors.

Non-viral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. The most

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common non-viral vector is probably the liposome. Liposomes are phospholipid vesicles capable of fusing with a cell membrane, thereby delivering their content, in our case, the expression plasmid containing the gene of interest, into the cell. Liposomes are advantageous because they are relatively cheap and nonpathogenic but, although they possess the highest transfection efficiency among the non-viral vectors their gene transfer efficiency is still significantly inferior to the one of viral vectors. [309] Other non viral method of gene transfer include the transfer of naked DNA, DNA coupled to polypeptides targeted to specific cell surface protein, DNA condensed with inactivated viral particles, crystals of calcium phosphate or coated microprojectiles. [309, 310]

Viral vectors are the most widely used because of their high transduction efficiency when compared to non-viral vectors. Viral vectors take advantage of the natural ability of the viruses to infect cells while avoiding the host immune response. The viruses used in gene transfer technology are rendered safe by the inactivation of one or more genes essential for their replication. [311] Among the most commonly used viral vectors are the adenoviruses, retroviruses, herpes simplex viruses (HSVs) and adeno-associated viruses (AAVs).

1.5.10.1 Adenoviral vectors

Wild-type adenoviruses are a large group of similar viruses that can cause respiratory and eye infecton in human.[312] There are 49 known serotypes (Ad1 –Ad49) and six distinguishable subgroups (subgroup A to F) [313] Serotypes 2 and 5 are the best characterized and the most commonly used for gene transfer. [314, 315]

Adenoviruses have a 36 kb, linear, double-stranded DNA genome with inverted terminal repeats at either end. The genome is packaged within an icosahedral viral capside that forms a nonenveloped virion with a diameter of approximately 140nm. The life cycle of the adenovirus does not include the insertion of the viral genome into the

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host chromosome. After infection, the viral genome resides in the nucleus of the host cell as an epsiomal element. Transcription, replication and packaging of the virus occur in the nucleus. The viral chromosome encodes eight polymerase II dependent transcription units that are referred to as early, intermediate and late with respect to the onset of their expression. [316] The early genes, E1, E2, E3, and E4, encode for multiple polypeptides important for the successful completion of the viral life cycle. The protein encoded by the E1 region of the virus are essential to activate transcription of the E2, E3 and E4 genes that encode for additional regulatory proteins and polypeptides important for viral replication. Late in infection, the major late promoter (MLP) is activated, resulting in expression of proteins important for packaging of the viral DNA.

Several versions of replication incompetent adenoviral vectors have been developed. [317-320] These adenoviral vectors can be divided into three general categories: first generation, second generation and gutless. These vector types differ in the numbers of viral genes that have been inactivated. First generation vectors typically have deletions in the E1 region of the genome rendering the virus replication deficient. Because viral replication is necessary to propagate vector stock, the deleted genes have been inserted in a 293 immortalized cell line of human embryonic kidney that supply the necessary E1 function in trans. [321] The E3 region of the viral genome is not necessary for in vitro virus propagation but one of its normal function is evasion of the host immune response which can be valuable asset for a vector, therefore in most first generation vectors only part of the region is deleted in order to allow greater cloning capacity. Typically viral vectors with E1 and E3 deletions have a cloning capacity of approximately 8Kb. [311] The biggest disadvantage associated to first generation vectors is the short length of transgene expression that is usually limited to weeks. [319, 322] The reasons for this are twofold. Because the viral DNA is not incorporated in the host chromosome and remains in the nucleus of the effected cells as an episomal element that is unable to

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replicate itself during cell division, the viral genome is not maintained in the nucleus of the infected cell and is eventually degraded. A low but significant amount of viral proteins are expressed after cell infection resulting in the stimulation of an immune response to the virally-infected cells in vivo allowing for the destruction and clearance of those cells. [323-325] Additionally, adenoviral proteins that remain in the viral vector preparations due to insufficient purification can also cause an anti-adenoviral immune response. [326] Re-dosing, with the viral vector, tissues that have lost transgene expression does not restore expression because of the humoral response mounted against the virus. [327] First generation adenoviral vectors have been used to deliver several marker and therapeutic genes to joints of numerous different animals including the rabbit, rat, mouse and horse. [328, 329, 330, 280] In horses, injection of 20 $X10^{10}$ particles into carpal joints resulted in transgene expression for up to 28 days. [280] Higher infection doses generated higher levels of expression but also exacerbated the immune reaction significantly decreasing the length of transgene expression. An intra-articular dose of 50 X 10^{10} particles shorten the transgene expression by 14 days. [280] The narrow window of transgene expression, the stimulation of the immune response and the inability to re-dose led to the development of second-generation adenoviral vectors and gutless vectors.

Second-generation adenoviral vectors were designed in the hope of decreasing the immune response of the host to the viral protein being expressed. Therefore the initial approach was to remove some or the entire reading frame of the E2 or E4 genes that encode transacting regulatory proteins. [331, 332] These second generation vectors are grown on 293 cells modified to express the polypeptides encoded by the E2 or E4 genes. Studies using these vectors indicate that they have reduced immunogenicity in vivo and extended expression of transgenes. [331, 333] Unfortunately it appears that those vectors also have a decrease in the level of transgene expression that seems to

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be associated to the E4 polypeptide ability to enhance gene expression from the viral genome. [333, 334]

An alternative approach to reduce the immunogenicity of the vector is to completely remove the viral genes, leaving only the inverted terminal repeats and the packaging site. [335, 317, 318] These gutted viruses are helper dependent in that propagation of the virus requires the co-infection with first or second generation viral vector in 293 cells. The helper virus provides the necessary adenoviral functions for replication and packaging of the gutless genome. The major advantages of these vectors are the absence of viral protein expression and the larger cloning capacity; up to 30 Kb of foreign DNA. Their major inconvenient is the difficulty to purify them from the helper virus particles.

The adenoviral vector used in the present study was created using the helper-dependent adenovirus vector system utilizing the Cre-mediated excision of the helper virus to create an E1/E3 deleted adenoviral vector. The Cre system is based on the P1 bacteriophage Cre-*loxP* system. The Cre gene encodes for a recombinase that facilitates the recombination between two parallel *lox* sites resulting in the excision of the intervening sequence, producing two recombination products each containing one *lox* site. In this case the helper virus is designated to have two *loxP* sites on either side of the packaging signal. When this helper virus is used in CRE8 cells, a modified 292 cell line making a high concentration of Cre recombinase, recombination will delete the intervening packaging sequence producing an unpackageable viral genome. When generating virus, the Ψ5 virus is used as a donor for the viral backbone. In the contest of the CRE8 cells there is negative selective pressure against the propagation of Ψ5 viral backbone. Furthermore, the Cre recombinase catalyzes recombination between Ψ5 and

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the shuttle vector with a single *loxP* site and a transgene, providing an efficient means to construct recombinants. [336]

1.5.10.2 Gene therapy and cartilage healing

The use of gene therapy for the treatment of osteoarthritis has been on the forefront of orthopaedic research for a decade and, numerous molecules with anti-arthritic potential have been tested in various animal models utilizing different vectors. [280, 337-341] Although the anabolic effects of various growth factors on cartilage metabolism are well known and have been studied in vitro as well as in some in vivo models, only a few research groups have investigated their use in gene therapy to enhance cartilage healing.

The first studies that were conducted in the use of gene transfer technology for treating cartilage injuries involved the use of ex vivo techniques and reporter genes. They were performed to ensure that defect implanted, genetically modified cells were able to express the transgene for a significant period of time. A study by Roessler et al. used an adenoviral vector carrying the β-galactosidase gene and primary rabbit chondrocyte cultures transduced in vitro. The transduced allogeneic chondrocytes were allowed to adhere to a type I collagen matrix for 7 to 10 days before being implanted in osteochondral defects created in rabbit trochlear grooves. Results showed that although lacZ-positive cells were still present at the surface of the implanted matrix 10 days following implantation, their numbers and drastically decline. Furthermore the greatest loss of cells (approximately 90%) was seen 24 hours after implantation of the matrix into the defect. The authors associated that lost to the mechanical forces of impact loading. [342] Another group used costal perichondral β-galactosidase transduced cells seeded into a biodegradable polylactic acid scaffold implanted into full thickness articular cartilage defect in the rabbit femoral condyle. Cells were transfected using a non-viral

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system and selected for β-galactosidase activity before being seeded in the matrix. As in the previous study transgene expression was mainly seen at the surface of the defect but there was no report of significant lost of expression 7 days after transplantation. [343] Grande *et al.* also studied the expression of the β-galactosidase gene in rabbit osteochondral defect implanted with periosteal-derived mesenchymal stem cells transduced with a retrovirus using an ex-vivo approach. There results show a stable expression of the transgene by the cells in the polyglycolic acid matrix for at least two months. [344] A fourth study also looking at marker gene expression in rabbit full thickness chondral defect implanted with transduced cells used the green fluorescent protein (GFP) gene instead of the β-galactosidase one. Allogeneic chondrocytes were transduced with a retrovirus and grown on poly (L-lactide) scaffold before being transferred to experimentally created cartilage defects. Contrary to the two previous but in accordance with the third study, expression of the transgene remained stable for a period of at least four weeks. The authors mentioned a high and homogeneous expression of GFP through out the implants four weeks after transplantation. [345]

The first report of the transfer of a therapeutic gene to joint tissue with the objective to improve cartilage healing was made by Grande *et al.* They used the same model described above but replaced the β-galactosidase gene with the human bone morphogenetic protein-7 gene. The group reported their results 8 and 12 weeks after implantation and compared them to empty defects or defects implanted with cells transduced with the neomycin gene. At the eight-week examination they noted that most of the knees with the BMP-7 gene-enhanced graft were completely healed with hyaline-like cartilage and that cartilage regeneration was observed to continue to improve up to 12 weeks in defects that were not completely healed after 8 weeks. In comparison non-treated defects (with or without cells) had not regenerated significant amount of cartilage. Histologic and immunohistologic evaluation confirmed their

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macroscopic impression. Treated defect presented with significant subchondral bone reconstitution underlying predominantly hyaline-like tissue that appeared analogous to the normal adjacent tissue and stain preferentially for type II collagen. Control defects had poor bone and cartilage regeneration, the repair tissue was mostly fibrocartilage and mostly expressed type I collagen. The authors did not mention any unwanted side effects related to their method or choice of growth factor. [346]

The only other report that studied the potential of gene therapy to enhance cartilage healing was done in a rat model of partial thickness defect using BMP-2 and IGF-1 as transgenes. The authors initially evaluated two different approached to induce transgene expression in the joint. The direct in vivo approach using an adenovirus successfully transduced cell of the synovial membrane, but also cells of the meniscus, collateral and cruciate ligaments with BMP-2 and IGF-1 as shown by formation of hyaline-like cartilage in those tissue. Direct injection also created substantial synovial membrane inflammation and a humoral response to the adenoviral vector. On the other hand ex vivo transduction of chondrocytes and fibroblasts using the same viral vector followed by intra articular injection of the cells showed newly form chondroid tissue in the synovial membrane but no inflammation or immune response. [347] Therefore the second approach was used and BMP-2 or IGF-1 transduced periosteal-derived mesenchymal stem cells were delivered to partial thickness defect in a fibrin glue. Three and eight weeks after treatment, empty control defects did not heal or contained cells and matrix debris. Defect filled with non-transduced cells either failed to fill the defect or to produce cartilage-like matrix and adjacent cartilage showed decrease staining for proteoglycans. Defect treated with either BMP-2 or IGF-1 transduced cells showed nearly completely filled defects with cartilaginous repair tissue that was clearly positive for type II collagen production and showed faint or no expression of type I collagen. The repair tissue in those defects also showed intense staining for proteoglycans with no loss

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of staining in the adjacent cartilage. Differences were noted between the defects treated with BMP-2 and IGF-1. The high proliferative potency of BMP-2 increased the number of cells present in the defect but also caused the formation of osteophytes in regions where cells seeded following dislodgement from the matrix. This side effect was not seen in IGF-1 treated defects but because of its lower proliferative effect on mature cells the cellular density and proteoglycan content was lower in those defects. [244]

1.6 Justification and rationale

If not treated full thickness chondral lesions often progress to osteoarthritis, a debilitating disease that affects many horses and costs the horse industry millions of dollars each year. Decreasing morbidity in animals and human suffering from the disease has been one of the focuses of arthritis research for quite some time. However clinicians and researchers working in the field are still looking for an effective and practical way to resurface cartilage lesions with repair tissue that possesses the biochemical and biomechanical characteristics of normal articular cartilage. In the last three decades a tremendous amount of information has been gained in the area of osteoarthritis pathophysiology and cartilage healing. Our knowledge of cytokines, enzymes and other factors involved in cartilage destruction, the anabolic potential of many growth factors, the great regeneration capacity of undifferentiated mesenchymal stem cells and the increased access to gene transfer techniques has helped us find and refine methods to enhance cartilage healing. But even though significant progress has been made, no one approach has been proven better than others at restoring a functional joint surface after articular cartilage has been damaged.

In the search for the ideal method to restore articular cartilage integrity it is important to remember to minimize patient morbidity, therefore our efforts should be directed at finding a minimally invasive technique that would allow for a fast recovery

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with the least chance of complications. Cost effectiveness is also an important factor in the development of new techniques, especially when we are taking about treating animals. With that said, focus should be placed on enhancing techniques that require only one surgical procedure and can be performed arthroscopically. If the local use of enhancing growth factors or other molecules is planned, they should be implanted at the time of surgery our shortly after by the use of a single intra-articular injection and, be present in the joint a therapeutic concentration for a relatively long period of time (weeks to months). Since most factors identified to have beneficial effects on cartilage metabolism are proteins and are therefore rapidly eliminated from the joints an effective way to maintain therapeutic concentration in the joint without having to perform daily intra-articular injections in the affected joint is necessary.

Previous research by our group has demonstrated that subchondral bone plate microfracture, a simple arthroscopic technique relying on the endogenous cell population present in the defect for healing was able to increase the quantity and the quality of the repair tissue present in experimentally created full thickness chondral defects. [182] One of our collaborators has shown similar results with the use of IGF-1 implanted in defects with the help of fibrin glue. [242] Our group has also demonstrated that the equine IL-1ra gene can successfully be delivered to equine joints by the use of an in vivo delivery system using an adenoviral vector and be expressed for a period of approximately 28 days. That expression translated into improvement in osteoarthritis parameters in joints with osteochondral fragments. [280] In vitro studies using a co-culture system with IL-1 depleted cartilage showed that the combined use AdEqIL-1ra and AdEqIGF-1 was able to have a partial restorative effects on cartilage PGs levels. [369] These results along with what is known on cartilage healing biology and, the criteria for an ideal technique to enhance cartilage healing led to the elaboration of this PhD project. The project combines the use of subchondral bone plate microfracture with

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direct gene transfer of the equine IL-1ra and IGF-1 genes to joints to enhance cartilage healing of full thickness chondral defect created in equine carpi and stifles.

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CHAPTER 2

2 Materials and Methods

2.0 Experimental design

Twelve mixed breed horses, 2 to 5 years of age were randomly divided into 2 groups. Eight horses were assigned to the treated group and 4 to the control group. All horses had full thickness a subchondral bone defect created on both distal radial carpal bones and medial femoral condyles (figure 1). Subchondral bone plate microfracture (SBPM) was performed on all the defects. The horses in the treated group had one randomly selected middle carpal joint and the contralateral medial femorotibial joint treated with an intra-articular injection of viral vectors carrying the equine IL-1ra and IGF-1 genes (AdEqIL-1ra and AdEqIGF-1) at the end of the surgical procedure. The opposing joints received the same volume of a placebo. The horses in the control group had all their operated joints injected with the placebo (figure 2).

Following surgery the horses were placed on complete stall rest for 8 weeks followed by 4 weeks of hand walking and 4 weeks of treadmill exercise. The horses were euthanized 16 weeks post surgery and healing of the defects was evaluated. Methods used to evaluate healing included: macroscopic, histologic and immunohistologic evaluations of the repair tissue, biochemical methods to measure glycoaminoglycan content, and polymerase chain reaction to measure mRNA expression of various molecules implicated in cartilage healing. Regular arthrocentesis of the middle carpal joint and synovial fluid analysis was performed to evaluate the

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length and level of expression of IL-1ra and IGF-1 as well as their effect on joint physiology. Potential clinical effects of gene therapy were evaluated by weekly determination of joint effusion, and by a mid-point and terminal lameness examination. Radiographs were also taken before surgery and at the end of the experimental period to assess possible bony changes associated with the creation of the defects and, or gene therapy.

2.1 Pre-operative procedures

Twelve mixed breed horses between 2 and 5 years of age were bought from a local vendor. Horses were stalled in 12' X 12' stalls bedded in wood shavings and allowed free access to grass hay and water. During a 10 day acclimatization period, blood work (CBC and chemistry panel) and a complete physical examination were performed on each animal to ensure that they were in good health, without palpable middle carpal and stifle joint effusion. During that same period horses were dewormed (Equvalan, PO) and immunized against Eastern/Western encephalitis, tetanus, influenza and rhinopneumonia. (Fluvac Innovator, IM). Each horse had their feet trimmed at the beginning of the project and 8 weeks after surgery.

2.2 Lameness evaluation

To ensure that the horses were free of lameness before and after joint manipulations, a lameness examination was performed on all horses at a trot. Baseline lameness was graded from 0 to 5 according to the guidelines established by the American Association of Equine Practitioners. [348] Carpal flexions were also performed for 45 seconds, after which time the horses were trotted in a straight line and the response to flexion graded from 0 to 4. A grade of zero indicated no change from baseline lameness while a grade of four indicated a severe increase in lameness. The

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examinations were recorded on a digital video camera for later evaluation. The lameness exams were also repeated at week 8 and before euthanasia, 16 weeks post surgery.

2.3 Radiographic evaluation

Radiographs of the both stifles (caudocranial and flexed lateral views) and carpi (dorsopalmar, lateral-medial, dorsolateral-palmar medial oblique, dorsomedial-palmar lateral oblique and flexed lateral views) were taken on each horse using a portable X-ray machine. The horses were sedated to facilitate the procedures using a combination of xylazine hydrochloride (0.2 to 0.5 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV), or detomidine (0.01 mg/kg IV). Horses with visible lesions in any of the joints were eliminated from the study. Radiographs were repeated on week 16 to evaluate the presence of articular changes following creation of the defects.

Radiographs were graded by a board certified radiologist unaware of the treatment assignments, for changes in the subchondral bone and for periarticular osteophyte formation. A grading scale from 0 to 4 was utilized to evaluate radiographic subchondral bone changes (0 = no significant changes, 1 = flat subchondral bone, 2 = defect in subchondral bone, 3 = lytic areas deep to subchondral bone, 4 = large lytic area deep to subchondral bone, cystic lesion) deep subchondral bone lysis with or without bone fragmentation). The extent of periarticular osteophyte formation was graded as nonexistent, mild, moderate or severe and were assigned a numeric value of 0 - 3 respectively.

2.4 Surgical procedure, Full thickness chondral defect creation and SBPM

The morning of surgery the horses were medicated with procaine penicillin (22,000 IU/kg, intramuscularly, q 12 hr) and phenylbutazone (4.4 mg/kg, orally, q 24 hr).

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These treatments were repeated for 5 additional days following surgery. Each horse was premedicated with xylazine (1 mg/kg, IV), induced with valium/ketamine (0.1 mg/kg, 2.2 mg/kg, IV) and anesthesia was maintained with halothane in 100% oxygen through a semi-closed breathing system. Assisted ventilation was used if the end tidal CO2 level rose above 45 mm Hg. Bilateral carpal and femorotibial arthroscopies were performed after sterile preparation of the surgical sites with the horses positioned in dorsal recumbency. The arthroscope portal in the midcarpal joint was located between the extensor carpi radialis and the common digital extensor tendon while the instrument portal was made medial to the common digital extensor tendon. [349] The femorotibial joint was accessed via a lateral approach with the arthroscopic portal situated caudal to the lateral patellar ligament, cranial to the long digital extensor tendon and just proximal to the tibial spine. The instrument portal was created medial to the middle patellar ligament and 1 cm proximal to the medial meniscus. [350, 182] Synovial fluid from both middle carpal joints was collected before the start of the procedure. Using a hand curette, a $1cm^2$, full thickness square chondral defect was created on the medial condyle of the femur and the weight bearing dorso-lateral aspect of the radial carpal bone. Using the same curette, the calcified cartilage layer was removed without disturbing the subchondral bone plate. During arthroscopy, custom made calibrated probes were used to confirm defect size to ensure defect uniformity. Subchondral bone plate microfracture was performed in all defects using an orthopedic bone awl with a 35° angled tip. Perforation of the subchondral bone plate was performed in a uniform manner to cover the entire surface of the defect, with spacing of 2 to 3 mm and depth of 3 mm. [182] After creation of the defects, the joints were lavaged, the arthroscopic portals were closed, and the randomly selected middle carpal joints and contralateral femorotibial joints were injected with 20×10^{10} particles of AdEqIL-1ra and 10×10^{10} particles AdEqIGF-1 diluted in 2 ml of Gey's balanced salt solution (GBSS). The opposing joints

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received the same volume of GBSS. The incisions were bandaged in a sterile fashion and the horse recovered.

2.5 Postoperative evaluation

Postoperatively the horses were monitored for general signs of discomfort such as depression, lost of appetite and lameness. Presence of swelling, heat and pain at the surgical sites was also evaluated. Clinical signs, if present, were treated with antibiotics and/or anti-inflammatory drugs following predetermined criteria assessed by the use of an orthopaedic pain score system. Humane end-points were established and all procedures and evaluation protocols approved by CSU Animal Care and Use Committee.

2.6 Exercise protocol

Following surgery the horses were put on stall rest for 8 weeks. Exercise began on week 9 post surgery. The horses were hand walked 5 days a week, 5 minutes/day with weekly increments of 5 minutes until they were walking for 20 minutes each day (total of 4 weeks). On week 13 post-operatively the horses were introduced to treadmill exercise. The exercise schedule consisted in 2 min of trot at 10 mph (4.45 m/s) for the first week, followed by 5 min of trot at 10mph for the last 3 weeks of the project.

2.7 Synovial fluid collection and Effusion scores

Collection of the synovial fluid from the middle carpal joints was performed on all horses at the time of surgery, every week for 6 weeks after surgery and every other week thereafter. Before collection the articular effusion of each joint was subjectively evaluated using the following grading system: non effusion = 0, slight effusion =1, mild effusion = 2, moderate effusion = 3 and severe effusion = 4. [351, 352] For synovial fluid collection, both knees were prepared in a sterile fashion with betadine and alcohol

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scrubs. Under sterile conditions and with the knee in flexion, a needle was inserted in the dorso-lateral aspect of the middle carpal joint and 2 to 6 ml of synovial fluid was aspirated using a 10 ml syringe. Sedation was administered as needed using the same doses as previously mentioned. Half a milliliter of synovial fluid was transferred to an EDTA tube for conventional synovial fluid analysis and the rest to a red top tube for IL-1ra, PGE_2 and IGF-1 quantification.

2.8 Conventional synovial fluid analysis

Synovial fluid contained in the EDTA tube was sent to the clinical pathology laboratory to be evaluated. Color, clarity and mucin content were evaluated in a subjective manner. Total protein concentration, white blood cell count and differential were determined by use of routine clinicopathologic methods.[i] Color of synovial fluid was determined to be yellow, colorless, straw, orange or red and assigned a numeric values of 1 – 5, respectively. Clarity was graded as clear, cloudy or hazy with numeric values 1,2 or 3 respectively. Mucin content was graded as good, fair or poor with respective numeric values of 1 – 3.

2.9 IL-1ra quantification

A commercial immunoassay [ii] using the manufacturer's recommendations was used for quantitative determination of IL-1ra levels in the synovial fluid. The assay employs the quantitative sandwich immunoassay technique. A mouse monoclonal antibody against E. Coli-derived recombinant human IL-1ra is pre-coated onto a 96 well microplate. Samples and standard are pipetted into the wells and any IL-1ra present is bound to the immobilized antibody. After a washing step to remove any unbound substances, a polyclonal, secondary antibody conjugated to horseradish peroxidase and specific for IL-1ra is added to the wells. Following a second washing step to remove any

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unbound antibody-enzyme reagent a substrate solution consisting in a 2 part-color reagent containing chromagen tetramethylbenzidine and hydrogen peroxide is added and color develops in proportion to the amount of IL-1ra present in the sample. The color development is stopped and the optical density is measured using a microplate [iii] reader set to 450 nm with wavelength correction set to 540 nm. The assay detection limit for biological fluid is typically less than 22 pg/ml. There is no significant cross reactivity or interference with factors related to IL-1ra or any of the most common recombinant human cytokines.

Synovial fluid collected in the red top vacutainer was centrifuged 5 min at 4°C at 3000 rpm. The resulting supernatant was aliquoted into 2 cryovials and stored at -80°C. To perform the IL-1ra quantification assay, one of the tubes was thawed a room temperature, 400 µl were used for the assay and 250 µl of each sample were set aside in a new, labeled tube to be send to the comparative orthopedic laboratory at Cornell University for IGF-1 quantification. The remaining synovial fluid was replaced in the -80°C freezers. Thawed samples were diluted 10, 100 or 1000 times with the RD6G diluent provided in the kit. Four hundred microliters of each sample was pipetted in individual wells of a 96 well microplate and the manufacturer's recommendations were followed for the remaining of the procedure. All samples were assayed in duplicate.

2.10 PGE_2 extraction

Because of its low level in synovial fluid PGE_2 has to be extracted before it can be adequately quantified using an immunoassay. PGE_2 extraction was performed on the synovial fluid stored in the second, never previously thawed cryovial. After thawing at room temperature, 500 µl of fluid was transferred to a 1.5 ml microcentrifuge tube to which 500 µl of 80% ethanol and 10µl of glacial acetic acid were added. The tubes were vortexed and then incubated 5 minutes at room temperature before being centrifuged 8

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minutes at 5500 rpm in a table tube centrifuge. The supernatant was transferred to C2 ethyl minicolumns [iv] placed under vacuum that had previously been washed twice with 1 ml of 10% ethanol. After addition of the supernatant the columns were washed with 1 ml of deionized water followed by 1 ml of hexane. The prostaglandins were eluted from the columns with a total of 1500 µl of ethyl acetate (2 X 750 µl). As a final step, the samples were placed in the multi-port evaporation system Savant Speed Vac Plus [v] for 1 to 2 hour to allow the complete evaporation of the ethyl acetate producing a yellow pellet at the bottom of the tube. The samples were stored at -20°C after the tubes were sealed with parafilm.

2.11 PGE_2 quantification

Again a commercial immunoassay [vi] using the manufacturer's recommendations was used for quantitative determination of PGE_2 concentration in the culture media. The assay is a competitive immunoassay that uses a monoclonal antibody raised against PGE_2 to bind, in a competitive manner, the PGE_2 in the sample or an alkaline phosphatase molecule that has PGE_2 bound to it. Quantification is possible by the measure of the intensity of the yellow color after the addition of the phosphatase alkaline substrate. The intensity of the color is inversely proportional to the concentration of PGE_2 in the samples and is detected with a microplate [iii] reader set to 405 nm with a wavelength correction set between 570 and 590 nm. The assay detection is typically 8.25 pg/ml. There is 70 % cross reactivity with PGE_1, 16.3 % with PGE_3 and less than 1 % with other related ecosanoid compounds.

The PGE_2 pellet was resuspended in 400ul of the assay buffer provided in the kit and the assay was performed according to the manufacturer recommendations. Note that some pellets were harder than others to resuspend and that in some cases there were small fragments of the pellet still visible in the buffer. All samples were assayed in

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duplicate and no dilutions were needed. Non-used resuspended samples were replaced at -20°C.

2.12 IGF-1 quantification

The concentration of IGF-1 in the synovial fluid of the middle carpal joints was determined by a disequilibrium radio-immunoassay performed in Dr Nixon's laboratory at Cornell University using a commercial kit from Nichols Institute.

2.13 Euthanasia and post mortem examination

At week 16 post-surgery all horses were euthanized according to the Colorado State University Animal Care and Use Committee guidelines. An overdose of intravenous sodium pentobarbital (Beuthanasia solution, 88mg/kg,) was used after the horses had been sedated with xylazine hydrochloride (0.5 to 1.0 mg/kg, IV) and placed in a chute built for this purpose. Each horse was photographed for identification purposes and, pictures of both carpi and stifles were taken. The limbs were labeled and both carpi and stifles were cut from the rest of the leg and sent for magnetic resonance imaging (MRI). Upon returning from imaging, the skin was removed from the limbs and the joints aseptically prepared before being opened in a sterile fashion for tissue collection and macroscopic evaluation.

2.14 Macroscopic evaluation

Once opened the joints were photographed including close up views of the defect and repair tissue as well as any other significant abnormalities. The repair tissue filling the defects was then evaluated in different categories and graded using the following grading system: none = 0, slight =1, mild = 2, moderate =3, marked = 4. The categories included: presence of irregularities at the surface of the repair tissue, relative firmness of the repair tissue, attachment of the repair tissue to the surrounding normal cartilage as

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well as to the underlying subchondral bone. A subjective evaluation of the area of the defect covered by the repair tissue and the volume of the repair tissue filling the defect was also performed. The presence of synovial membrane adhesions and lesions on other bones or structures of the joints were recorded.

2.15 Sample collection

Synovial membrane, normal cartilage adjacent to the defects, repair tissue filling the defects, sections of subchondral bone with repair tissue or, adjacent normal cartilage as well as section of third carpal bone with kissing lesions were collected and stored as pertained by experimental protocols of the assays performed on the tissues (figure 3). Samples of tissue destined for PCR were placed in 1 ml of TriZol reagent and stored at -80°C. Tissue samples that underwent biochemical analysis to determine collagen and glycoaminoglycan content were placed in an empty cryovial and stored at -80°C. Specimens for histologic evaluation were placed in a labeled cassette and plunged in a jar of 10% formalin. Tissue for in situ hybridization and immunohistochemistery were snap frozen in OCT compound [vii] wrapped in aluminum foil and stored at -80°C.

2.16 Glycosaminoglycan (GAG) quantification

The amount of GAGs present in the repair tissue and the adjacent normal cartiage was determined by the use of the dimethylmethylene blue (DMMB) colorimetric assay. The dye 1.9-dimethylmethylene blue possesses strong metachromatic properties and binds to the GAG anionic groups. The first step in GAG quantification involves the digestion of cartilage with papain to break down the proteoglycans of the extracellular matrix into GAGs. This preliminary step also helps to eliminate part of the other anionic groups that might interfere with GAG quantification.

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The cartilage samples were thawed at room temperature and weighed. Pieces greater or equal to 5 mg wet weight were place in a microcentrifuge tube and desiccated over night in the multi-port evaporation system Savant Speed Vac Plus.[v] The dry weight of each sample was recorded and 100 µl of digestion solution / 1 mg of dry tissue was added to each tube. The tubes were then placed a 60°C for an overnight digestion (approximately 18 hours). Some of the repair tissue samples were not completely digested after 18 hours; therefore they were replaced at 60°C for an additional 4 hours, under gentle agitation to complete the digestion process. The digestion reaction was stopped with the addition of 2 µl of 200 mM iodoacetate for each 100 µl of digestion solution. If not used immediately, the samples were stored at -80°C until used. Short term refrigeration and storage at -20°C (24 hours or less) is also a viable option and, will not affect the GAGs present in the samples.

To perform the DMMB assay, 10 µl of digested samples were placed in wells of a 96 well U-bottom plate and 190 µl of dye solution was added. The plate was read immediately at 530nm in a Dynex MRX plate reader.[iii] The standard curve use for the assay was made from a 1mg/ml stock solution of shark chondroitine-sulfate dissolved in deionized water. The following concentrations were utilized; 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 150 mg/ml.

2.17 Histologic evaluation of the synovial membrane

Synovial membrane and joint capsule were harvested from each operated joint, fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H & E). Five micron sections were evaluated and graded blindly by 3 evaluators for cellular infiltration, synovial intimal hyperplasia, subintimal edema, subintimal fibrosis and vascularity. [351,352] Each variable was graded 0 to 4 (0 = normal, 1 = changes in

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< 50% of the section, 2 = changes in approximately 50% of the section, 3 = changes in > 505 of the section, and 4 = changes throughout the section).

2.18 Histomorphometric evaluation of the repair tissue

Osteochondral sections from the defect and the repair tissue fixed in 10% formalin were decalcified and embedded in paraffin. Five micron sections were cut and stained with H &E.

The histomorphometric analysis of the H & E sections was done using an inverted microscope [viii] coupled to a computerized digital analysis software program. [ix] Multiple variables were evaluated to assess healing and the overall quality of the repair tissue. These included total area of defect, percent of repair tissue filling the defect, percent of fibrous tissue, fibrocartilage, hyaline-like cartilage, bone and percent of reactive cartilage/bone present in the repair tissue. Were also measured, percent of attachment of the repair tissue to the normal adjacent cartilage and subchondral bone, and percent of remaining calcified cartilage. Bone porosity underneath the defect and in an area under normal articular cartilage immediately adjacent to the defect was also estimated. The percent of bone porosity was defined as the portion of the measured area with no bony material divided by the total measured area times 100. [353]

2.19 Immunohistochemistry (IHC)

IHC was used to determine the localization and the abundance of proteins of interest in the different layers of the repair tissue. The basis behind IHC is the same as in immunoassays i.e. it relies on the principle of antigen recognition by antibodies but, in this particular technique antigens are located on histologic sections rather than in solution. Our laboratory uses an automated system (Nexes IHC [x]) that utilizes an indirect biotin avidin method (Ventana Basic DAB detection kit [xi]) to detect the antigen of

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interest. A primary antibody bound to the antigen is recognized by a biotinylated secondary antibody. Because of its high affinity to biotin the avidin section of an avidin–horseradish peroxidase conjugate binds to the biotin on the secondary antibody after its addition to the system. The avidin-enzyme complex is than visualized by the addition of diaminobenzidine (DAB) an electron donor and hydrogen peroxide, the enzyme substrate. The enzymatic reaction creates a brown precipitate visible under light microscopy.

Antibodies that recognized aggrecan, the protein S100, type I and II collagen were used for the immunolocalization of their respective peptide. The mouse monoclonal antibody AN9P1[xii] which recognizes keratan sulfate chains in aggrecan bound to the core protein was used at a 1:50 dilution in TBS. The mouse monoclonal antibody II-II6B3[xiii] specific for type II collagen was used at a 1:10 dilution in TBS. The mouse monoclonal antibody BYA65201[xiv] specific for type I collagen was used at a 1:100 dilution in TBS. The rabbit antibody Z0311[xv] specific for the calcium bounding protein S100 was used at a 1:200 dilution. Each antibody was tested for specificity and cross species reactivity before being used in this study. Normal horse serum was used as the blocking agent for type I collagen, aggrecan and S100 antibodies. Isolated type II collagen was used as the blocking agent for the type II collagen antibody.

Five micron frozen serial sections of repair tissue were cut from OCT embedded samples using a cryostat[xvi]. The section were placed on positively coated slides and fixed in acetone for 10 minutes. The slides were allowed to dry at room temperature before being placed a 4°C until further use. Slides can be stored that way up to a week before being stained. For immunochemical staining, slides were warmed to room temperature and washed in APK wash solution for 5 minutes. They were then placed in the automated stainer where they were washed 3 times with 500µl of APK wash solution. One hundred microliters of an inhibitor solution containing 2% hydrogen

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peroxide was then applied to the slides for 4 minutes. The slides were then rinsed with 500 µl of APK wash solution and incubated with 100 µl of chondroitanase ABC (2.5 U/ml) for 30 minutes. The slides were rinsed again and 100 µl of primary antibody or 100 µl of primary antibody with ten-fold excess blocking agent were applied for a period of 16 minutes. After another rinse, slides were incubated 8 minutes with 100 µl of the biotinylated secondary antiboby followed by a wash and 8 minute incubation with 100 µl of avidin-horseradish peroxidase. Localization of the antibodies in the tissue was performed by the addition of 100 µl of DAB hydrogen peroxide substrate solution for 8 minutes following a rinse with APK solution. Slides were then exposed to 100 µl of copper sulfate solution for 4 minutes to stop the enzymatic reaction and then counterstained with hematoxylin and a bluing agent. To minimize the evaporation of the aqueous reagents used in the procedure, 500 µl of an oil based liquid coverslip was applied between each step. Once counterstained the slides were removed from the automated station and washed in 100% ethanol for 2 minutes. They were then rinsed twice in xylene for 1 minute and a xylene based coverslip was applied.

The stained sections were evaluated by 3 individuals unaware of treatments. Each section was divided in three equal zones: superficial, middle and deep. Each zone was then graded from 1 to 4 for the intensity of positive staining for a specific antibody compared to a section stained with a control antigen/antibody mixture. (0 = no staining, 1 = slight staining, 2 = mild staining, 3 = moderate staining, and 4 = intense staining). Staining for S100 was graded as being positive or negative for each zone of the repair tissue.

2.20 TaqMan Real time quantitative polymerase chain reaction (PCR)

Real time quantitative polymerase chain reaction was used to quantify the mRNA expression of molecules of interest in cartilage healing. The ABI Prism 7000 Sequence

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Detection System [xvii] was used. PCR quantification using the ABI Prism 7000 system is based on the continuous measurement of PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe and is made possible by the exonuclease activity of the Taq DNA polymerase. The TaqMan probe, as it is called, is composed of a short oligodeoxynucleotide sequence (20-25 bases) homologous to an internal target sequence present in the gene of interest. The probe is labeled with two different fluorescent dyes, on the 5' end there is a reporter dye (6-carboxyfluorescein or FAM) and on the 3' end a quencher dye (6-carboxy-tetramethyl-rhodamine or TAMRA). When the probe is intact, energy transfer occurs between the two fluorophors and emission from the reporter is quenched due to the spatial proximity of the dye. Since the probe sequence is located between the two PCR primers and has a melting temperature 10°C higher than the primers it anneals first to the DNA allowing, during the extension phase of PCR, the probe to be cleaved by the 5' – 3' exonuclease activity of Taq polymerase thereby releasing the reporter dye from the oligonucleotide-quencher and producing an increase in fluorescence emission which is proportional to the amount of PCR formed. The ABI Prism 7000 uses a light source to excite each well and a CCD camera measures the fluorescence spectrum and intensity from each well to generate real-time data during PCR amplification. The software examines the fluorescence intensity of reporter and quencher dyes and calculates the increase in normalized reporter emission intensity over the course of the amplification. The results are then plotted versus time, represented by cycle number, to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches the threshold (defined

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as the threshold cycle number or CT). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube.

The mRNA expression of the extracellular matrix molecules aggrecan, type I and type II collagen was evaluated in samples of repair tissue as well as in synovium and normal cartilage adjacent to the defect. The same tissue samples were evaluated for the mRNA expression of the cytokines IL-1α and TNFα, the growth factor IGF-1 and the anti-inflammatory protein IL-1ra. The level of expression of MMP-3 and MMP-13, two metalloproteinases involved in cartilage degradation was also evaluated. The expression of the house keeping gene GAPDH was used as the internal control. Primers and probes specific for the equine sequence of most of these molecules (GAPDH, IGF-1, IL-1ra, Type 1 and type II collagen, IL-1α, TNFα, and MMP3) were designed, tested and bought from Dr Christian Leutenegger at the University of California, Davis [xviii]. The Aggrecan and MMP13 primers and probes were designed in our laboratory, synthesized by Applied Biosystems [xix] and tested for specificity before being used in this project.

2.20.1 RNA extraction (precautions)

RNA is a very labile molecule and care most be taken to prevent its degradation by RNases. Therefore, through out the extraction process gloves were worn at all time, samples were kept on ice when not handled, and all plastic and glassware were RNase free. RNases can be removed from glassware by baking it at 180°C overnight or by treating it for 1 hour in freshly prepared 0.1% diethylpirocarbanate (DEPC), draining and autoclaving it to destroy any unreacted DEPC. DEPC reacts with histidine residues of proteins and inactivates RNases. An alternative to DEPC treatment is the use of RNase Away which was used to treat surfaces, pipettors and other instruments that were needed for the extraction procedures. Solutions must be bought or made with RNase

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free reagents and DEPC treated water. Pipette tips and plastic tubes can be bought RNase and DNase free.

2.20.2 RNA extraction

First, synovium, normal cartilage or repair tissue samples stored in TriZol reagent were transferred to individually wrapped, sterile 15 ml culture tubes containing 1 ml of TriZol, then the samples were homogenized using the Cyclone Vitishear Homogenizer [xx]. If samples were not completely homogenized after one cycle, they were placed on ice to cool down and homogenized again, repeating the procedure until only small morsels of tissue were visible in the solution. The homogenate was transferred to a 1.5 ml microcentrifuge tube and stored at -80°C. To reduce chances of RNA degradation samples were homogenized 12 at a time and kept on ice throughout the procedure. For practicality reasons, all cartilage and synovial membrane samples were homogenized before the rest of the extraction procedure was performed.

The frozen homogenized samples were thawed and then incubated at room temperature for 5 minutes. Two hundred microliters of chloroform per ml of TriZol were added to each tube, the tubes were then vortexed and incubated at room temperature for 3 minutes and centrifuged at 12 000 x g for 15 minutes at 4°C. The aqueous phase containing the RNA was transferred to a new tube and the chloroform extraction was repeated using 1 volume of chloroform per volume of sample. The second extraction was performed to increase the purity of the RNA by removing more proteins from the homogenate. The aqueous phase from the second extraction was again transferred to a new tube and 500 µl of isopropanol per ml of TriZol was added, the tubes were mixed well and stored at -20°C for 2 hours to allow the RNA to precipitate. The RNA was pelleted by 30 minutes centrifugation a 12 000 x g at 4°C, the supernatant was removed and the RNA pellet was washed once in 1 ml of ice cold 70% ethanol. Following

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centrifugation at 7500 x g at 4°C for 5 minutes, the ethanol was removed with care (the pellet can easily detach and be aspirated) and the pellet dried under the fume hood for 5 to 10 minutes. Once dried the pellets were resuspended in RNase free water.[xxi] The volume used to dissolve the RNA varied with the size of the pellet. If the pellet was visible and of moderate size, it was resuspended in 30 µl, if the pellet was large, the volume was increased to 50 µl, and if the pellet was small or not visible the volume was decreased to 20µl. All the synovial RNA samples had a visible pellet and were therefore resuspended in 30 µl of water. Most of the RNA from the repair tissue had no visible of very small pellet and were resuspended in 20µl of nuclease free water. The RNA extracted from the normal cartilage had the bigger pellets, especially the stifle samples, and most of them were resuspended in 50 µl of water. It is important to note that most of the samples with moderate to large pellets were very hard to dissolve and had the consistency of gelatin. This can be attributed to the presence of DNA or other impurities like GAGs. Treatment with 1 µl of RNase free DNase for 20 minutes at 37 °C followed by 5 minutes of inactivation at 95°C and immediate cool down on ice helped considerably in the matter as well as allowed degradation of genomic DNA that could interfere with the PCR reaction. The RNA samples were stored at -80°C until needed.

2.20.3 cDNA synthesis

The synthesis of complementary DNA was done by adding 2 µl of random primers (300 ng / µl) to 10 µl of RNA and incubating the mixture at room temperature for 10 minutes. This step was followed by the addition of 8 µl of master mix (4 µl 5X first strand buffer, 1 µl 10mM dNTPs, 1 µl 0.1 M DTT, 0.5 µl RNase Out, 1 µl nuclease- free water, 0.5 µl Superscript II RNase H- Reverse Transcriptase) and a 50 minutes incubation at 42°C. The reaction was stopped by placing the tubes 5 minutes at 95°C then immediately cooling them on ice. The cDNAs were stored at -20°C until needed.

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2.20.4 TaqMan real time quantitative PCR

Five microliters of each cDNA samples were loaded on 96-well microtitre plate [xxii] kept on ice. To each one was added 12.5 µl of TaqMan Universal Mastermix, 0.5 µl of a 20 µM solution of forward primer, 0.5 µl of a 20 µM solution of reverse primer, 0.2 µl of a 10µM solution of TAMRA labeled probe and, 6.3 µl of nuclease-free water. The plate was vortexed, then briefly centrifuged before being placed in the ABI prism 7000 Sequence Detection System. The DNA amplification sequence was divided in three stages: 1 minute at 50°C followed by 10:15 minutes at 95°C and 1 minute at 60°C. This sequence was repeated 40 times for the synovium and normal cartilage samples, while it was increased to 50 cycles for the repair tissue samples in an effort to defect genes with low copy numbers in those samples with less RNA.

2.20.5 Conversion of the raw data

The CT value obtained for the GAPDH of each sample was subtracted from the CT value of all the other molecules amplified for that sample. The value obtained, the ΔCT, was average for the carpus and stifle locations in normal cartilage for each molecule. The averaged ΔCT for carpus and stifle was subtracted from their respective location ΔCT in synovial membrane, repair tissue and normal cartilage for each one of the molecule studied. The value obtained, the ΔΔCT, was entered in the following equation, $2^{-\Delta\Delta CT}$ to calculate the fold difference. That final value can be interpreted to be for each molecule, the fold difference between a particular sample and the average of the normal cartilage carpus or stifle value.

2.21 Statistical analyses

The data and results were subjected to two different analyses using a mixed model analysis of variance.[xxiii] The first analysis looked at the continuous dependent

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variables collected at multiple time points following creation of the defects. Those included clinical scores of lameness, joint effusion, IL-1ra, IGF-1 and PGE_2 synovial fluid concentrations, and synovial fluid cytology. Independent variable for the analysis included treatment effect (control, placebo and treated joints), time effect (weeks after defect creation the measurement was obtained), location effect (middle carpal of femorotibial joint location) and all the interaction between treatment, time and location. Horse was considered as an independent variable that was a random effect. The second analysis looked at the dependent variables that were collected at the termination of the project or at one time point. Those included radiographic scores, all parameters of gross and histomorphometric examination, synovial membrane evaluation scores, scores for type I, type II and aggrecan staining, repair tissue and adjacent normal cartilage GAGs and water content and, PCR results. This second analysis included the same independent variables and interactions as the first one with the exception of the time effect. When specific comparisons were made, a Least Squares Mean procedure was utilized. Main independent variable effects or the highest interaction of independent variables were reported for p values < 0.05. Results were reported as the mean +/- the standard error of the mean (Mean +/- SEM) unless otherwise noted.

Linear regression analyses were performed on the remaining length of calcified cartilage compared to the length of repair tissue attached to the subchondral bone, on the percentage of remaining calcified cartilage compared to the length of repair tissue attached to the subchondral bone, on the length of repair tissue attached to the subchondral bone and the total amount of repair tissue found in the defect, as well as on attachment of the repair tissue to adjacent cartilage and the amount of repair tissue present in the defect. Results were reported as a squared correlation coefficient (R^2)

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The categorical scores for S100 immunostaining were analyzed using a Chisquare test for homogeneity of proportions. Differences were considered significant whit a p-value < 0.05

Randomized residual plots were constructed for dependent variable data to test for normal distribution and equal variances with a mean of zero; if deviations from these assumptions were found the variable were transformed in order to fulfill the afore mentioned criteria and results were reported in their transformed form.

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CHAPTER 3

3 Results

3.0 Surgical model

Defects were created in all joints with minimal difficulty. Correct placement of the arthroscope portal was crucial for a good visualization of the defect site and ease of defect creation. Avoiding hemorrhage also prevented loss of visibility. In radial carpal bone defects, care needed to be taken when making the microfracture not to chip the free lateral edge of the distal radial carpal bone. Condylar defects had significantly more microfracture perforations (17.29 ± 0.60) than radial carpal bone defects (11.16 ± 0.60). Osteochondral fragments approximately 1 mm^2 were inadvertently created and removed from three radial carpal bone defects (two in the control group and one in the placebo group).

3.1 Clinical examinations

3.1.1 Lameness evaluation

Most horses were sound at their pre-operative lameness exam although some showed a slightly perceivable lameness at a trot (grade 1/5) on one of the four limbs. Taken all together, the baseline degree of lameness was the same for all horses in both carpi and stifles (figure 4).

Post operatively two horses became lame enough to necessitate cessation of exercise. One horse developed a foot abscess on week 12 and was grade 4 out of 5

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lame on the left front foot. The horses was treated and rested for 4 days before hand walking was resumed. Another horse injured both of his front superficial digital flexor tendons during carpal arthrocentesis on week 12 and became grade 4 out of 5 lame on the right front limb. The horse was treated and rested for 11 days delaying the start of treadmill exercise for one week.

Lameness evaluation at 8 weeks post defect creation showed a significant increase in front limb lameness but not in hind limbs. At the end of the study, the final lameness evaluation showed a significant increase in lameness in both front and hind limbs when compared to baseline. Although the severity of lameness was greater in front limbs at both time points it was only significantly different from hind limb lameness at the end point lameness evaluation (figure 4). Severity of lameness was not affected by treatment with EqAdIL-1ra and EqAdIGF-1 or the placebo (figure 5).

Carpal flexion tests were performed on all horses at week 8 and 16 post surgery. Lameness was not significantly affected by carpal flexion and the response to flexion was no different in control, placebo or treated joints. Furthermore, the response to the flexion test did not change with the increase in lameness severity seen between the time points.

3.1.2 Synovial joint effusion

Surgical creation of defects induced synovial effusion in both carpi and stifles that persisted throughout the duration of the project. Other than for weeks 1, 12 and 16 when the effusion scores were the same, effusion was found to be significantly greater in carpi than in stifles (figure 6). In general, the effusion scores for carpi and stifles stayed similar during the first half of the project (from week 1 to 8) followed by a significant decrease observed during the second half. There was no significant difference between the effusion scores of control, placebo and treated joints.

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3.1.3 Radiographic evaluation

The preoperative radiographs showed no observable lesions in the carpal and stifle joints of all the horses. Sixteen weeks following surgery and treatment with the viral vectors or the placebo, joints showed on average slight to mild radiographic changes. Lesions were classified in two categories, subchondral bone changes and periarticular osteophyte formation. Subchondral bone change scores were similar between carpi and stifles as well as between control, placebo and treated joints. Changes, when present (8/24 carpi, 8/24 stifles) were mostly associated with flattening of the subchondral bone. One treated stifle had a more severe lesion classified as a lytic area deep to the subchondral bone (score = 3 out of a possible 4). Osteophyte formation was dependent of both location and treatment. Although all groups and both locations had very mild changes on only a small number of joints (6/24 carpi, 4/24 stifles), there was significantly less osteophyte formation in the treated carpal joints than in the placebo carpal joints but, there was no difference between the treated carpal joints and the control ones (figure 7). A similar difference was not seen in the stifle joints. The combined score for subchondral bone changes and osteophyte formation was not statistically different between treatment groups or locations.

3.2 Synovial fluid analysis

3.2.1 Synovial fluid color, clarity and iatrogenic blood contamination (IABC)

Synovial fluid collected by arthrocentesis was generally yellow or straw colored and was clear to cloudy after centrifugation. Treatments and weeks into the project at the time of collection did no affect those parameters. Iatrogenic blood contamination was low at week 0 when arthrocentesis was performed under general anesthesia. Blood

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contamination increased when the collection of fluid was done on standing, sedated horses but, the frequency of its occurrence remained relatively the same throughout the project (figure 8). IABC was more frequently seen in joints that had been treated with the viral preparations than the contralateral, placebo treated joints. However there was no significant difference in occurrence between the treated joints and the joints of the horses in the control group that were also injected with the placebo (figure 9).

3.2.2 Synovial fluid mucin clot

Quality of the synovial fluid mucin clot was the same for all groups prior to surgery. Defect creation and treatment significantly decrease the quality of the mucin clot for all 3 groups (increased score). Decrease in quality was not statistically different between groups for the first 2 weeks after surgery. Mucin clot quality of the synovial fluid of the control joints returned to normal by week 3 and remained at that level for the remainder of the study. On the other hand, joints in the treated group and the placebo group took significantly longer to go back to baseline quality. Return to pre-surgical level was not seen, in both groups, until week 8 (figure 10).

3.2.3 Synovial fluid total protein concentration

The pre-surgical synovial fluid concentration of total protein was the same for all carpal joints. One week after surgery and treatments with viral preparations or placebo there was a significant increase in total protein concentrations in all joints when compared to baseline. The increase seen in the joints treated with AdEqIL-1ra and AdEqIGF-1 was significantly greater than the increase seen in the placebo and control joints. Even if the concentration of total protein was higher in the placebo joints than in the control joints, the difference was not statistically significant (figure 11). The concentration of total protein peaked at week 1 for all joints; it was followed, again in all

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joints, by a gradual decrease back to baseline. Although there was a significant decrease in the total protein concentration of the treated joints at week 2, the concentration remained significantly higher than the one seen in the control group where the level had gone back to baseline. A decrease in protein concentration was also seen in the placebo joints but it was not considered statistically significant from the previous week; the concentration remained above baseline and statistically greater than the one in the control joints. The total protein concentration in the treated joints remained elevated until week 5 when they dropped back down to baseline level. It took three weeks for the placebo joints to return to baseline concentration, while it took the control joints only one week. Overall, a significant increase in total protein concentration was seen after surgery and treatments, those concentrations were significantly higher in the treated joints and decreased significantly slower than in the control joints.

3.2.4 Synovial fluid white blood cell (WBC) count and differential

Surgical creation of the defects did not induce an acute or chronic increase in the WBC count since both placebo and control carpi WBC count remained at, or lower than baseline throughout the study. On the other hand, intra-articular injection of the viral preparations in treated carpi initiated a cellular response evident by the increase in the WBC count seen in the week following surgery. In that particular group, the WBC remained elevated for 2 weeks after surgery and returned to baseline on week 3 (figure 12). A differential performed on the synovial fluid showed that lymphocytes were mostly responsible for the increased cell count seen in the gene therapy treated joints and, although the WBC count had returned to normal, the lymphocyte count remained elevated for 8 weeks following surgery (figure 13). Such a phenomenon was not observed in the placebo and control carpi where the lymphocyte count remained below or equal to baseline at all time points. The rest of the differential showed that over all

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time points, the neutrophil count was significantly lower in the treated and placebo carpi than in the control ones (figure 14). It also showed that the monocyte count was lower than baseline in all the groups starting 2 weeks following surgery until the end of the study except for week 4 and 14 when it barely climbed back to baseline (figure 15).

3.3 Synovial fluid IL-1ra concentration

Above baseline IL-1ra concentrations were detected in the gene therapy treated carpi for 3 weeks after injection, with the highest concentrations seen one week after treatment. Values above baseline were not seen in the control carpi. However, there was a slight but significant increase above baseline levels in placebo treated joints one week after injection but the concentrations remained well below the ones obtained in the treated joints. (figure 16)

3.4 Synovial fluid IGF-1 concentration

Concentrations of IGF-1 in carpal synovial fluid increased significantly in all groups 1 week after defect creation. Concentrations remained elevated up to 6 weeks after surgery but analysis of the results showed no significant difference between the levels seen in the three treatment groups (figure 17).

3.5 Synovial fluid PGE_2 concentration

As for other synovial fluid parameters the baseline concentration of PGE_2 was the same for all carpi. Defect creation and treatment significantly affected PGE_2 concentration in all three groups. One week post-operatively PGE_2 concentrations were significantly elevated in all joints. The peak PGE_2 concentrations were seen at weeks 1 and 2 post-surgically for all groups. At those two time points, the PGE_2 concentrations of the treated joints were significantly greater than those of the placebo and control joints, while the level of those two last groups were not significantly different (figure 18). After

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week 2, there was significant decrease in PGE_2 synovial fluid concentration in both the treated and placebo joints while a significant decrease was not seen in the control joints until week 4. The difference in the PGE_2 concentration between the three groups of joints disappears by week 4 but all levels remained above baseline. A decrease below baseline was not seen in the treated joints until week 10 post surgery, while concentrations fell back to normal at week 5 for the control and placebo joints although there was a significant, short lived decrease to baseline on week 3 in the placebo treated joints.

3.6 Post mortem evaluation

Four months after its surgical creation, the defect site was still visible in all joints and presented with various degrees of healing. The repair tissue present in all defects was either white or clear colored or a combination of both. The color of the repair tissue of carpal defects was mostly white, while the color of the repair tissue present on femoral condyles was more a combination of white and clear. Thin areas of repair tissue tended to be clear while thicker areas were whiter. In some defects there were red, focal areas associated to either vasculature (3 carpi and 3 stifles) or hemorrhage (1 carpus and 1 stifle) (figure 19 and 20). No defect presented with an entirely regular surface and, surface uniformity was independent of treatment and location although there was a trend toward carpal defects having a smoother surface then condylar ones. (p-value = 0.0565, table 1) Surface irregularities ranged from being almost completely absent (figure 21) to the presence of numerous lumps and bumps as well as depressions and pits. The depressions and pits seem to be more common in the stifles and correspond to microfracture holes that had not filled in (figure 22). Thickness in the repair tissue also varied widely within and in between defects. Some were filled with a smooth layer of thick white tissue as the one in figure 21, while others contained only a thin layer of

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almost translucent tissue (figure 22). Within one defect this combination was also possible.

Indentation of the repair tissue with tissue forceps was used as a subjective way to evaluate tissue firmness. Analysis of the results showed that placebo treated defects (joints opposite the treated joints) had significantly firmer repair tissue then control defects but that there was no significant difference between treated defects and control defects. However treated defects firmness scores were higher than control ones (table 1).

Attachment of repair tissue to the surrounding normal articular cartilage was determined visually and with the aid of tissue forceps. Repair tissue from condylar defects (score = 2.58 ± 0.25) were determined to have better attachment to adjacent normal cartilage than repair tissue present in carpal defects (score = 1.88 ± 0.25). In most carpal defects, repair tissue did not reach the border of the defect while condylar defects tended to heal with more tissue around the border. Attachment to cartilage was not affected by treatments (table 1).

Two control carpal defects and one treated condylar defect had a soft central area that was completely detached from the subchondral bone and had the appearance of a bubble (figure 20). One control carpal defect had a small flap of repair tissue on its palmar lateral aspect that could easily be elevated. Some other carpal defects, regardless of treatments, showed area of poor attachment to subchondral bone when probed with tissue forceps. These areas were usually associated with poor attachment to adjacent normal cartilage. On average, repair tissue was well attached to the underlying subchondral bone (table 1). Subchondral bone attachment was independent of both location and treatment.

A subjective evaluation of the defect area covered by repair tissue showed that the surface covered by repair tissue was the same regardless of location and treatment

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(tables 2 and 3). However this measurement did not take into account the thickness of the repair tissue within the defect and, defects with thin repair tissue covering the whole surface of the defect received a better grade than defects with thick repair tissue present in only one area. The volume of repair tissue filling the defect was therefore evaluated. Analysis of those results revealed that on average, radio-carpal defects were filled with repair tissue up to 75.92 ± 5.82% of there original volume while femoro-condylar defects were filled with significantly less tissue (51.58 ± 5.82, p-value = 0.0004).

An overall subjective evaluation of healing, done by adding the scores of each of the previous parameters was calculated to give a general healing score. The results showed that healing of defects was the same regardless of if they were created on the radial carpal bone or in the medial femoral condyle and that healing was not affected by treatment with AdEqIL-1ra and AdEqIGF-1. However pattern of healing differed depending on location. Carpal defect tended to heal from the center toward the edges of the defect with little evidence of matrix flow, while condylar defects had a tendency to heal with a significant amount of matrix flow often leaving the center of the defect with no or very little repair tissue.

Synovial membrane adhesions were noted in one treated and one placebo carpus. Both were small and easily reduced, located adjacent to the defect on the dorso-lateral aspect of the joint. Kissing lesions were present in all the carpi and 13 of the 24 stifles. Carpal kissing lesions were located on the radial facet of the third carpal bone, in stifles they were seen on the medial tibial plateau abaxial to the intercondylar eminence. Lesions ranged from simple articular cartilage edema (17/24 carpi) that presented like yellow, rough and thickened cartilage to full thickness erosions to the level of the subchondral bone. By far, edema was the most frequent kissing lesion associated to the presence of the defect (17/24 carpi and 9/13 stifles). Partial thickness erosions came in second (9 / 24 carpi and 3/13 stifles) and the rest was separated between

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fibrillation and full thickness erosions. Although most of the cartilage distant to the defect sites appeared normal, lesions were found on the fourth carpal bone, the ulnar carpal bone, the intermediate facet of the third carpal bone, the lateral tibial plateau and the medial femoral trochlea.

3.7 Histologic evaluation of the synovial membrane

Histologic evaluation and grading of the changes present in the synovial membrane of control, treated and placebo joints showed various degree of changes in all joints. Cellular infiltration was noted to be higher in the treated joints (1.63 ± 0.28) when compared to control (0.81 ± 0.32) and placebo joints (1.00 ± 0.28) but the difference was not significant (p-value = 0.06). The infiltration was characterized mainly by focal areas of inflammatory cells, mainly lymphocytes, present in the subintima or around the vessels (figure 23). A more diffuse type of infiltration was also occasionally seen. The degree of intimal hyperplasia was more severe in the synovial membrane of the carpal joints (1.54 ± 0.13) than in the stifle joints (0.25 ± 0.13) independent of treatment. In the hyperplastic regions, the intimal layer was thickened and was composed of a three to four cell layer instead of the regular one or two (figure 24). Subintimal edema was absent from all but two control carpal synovial sections and was graded as slight and mild. Overall subintimal fibrosis and increase in vascularity were slight to mild and were similar in all treatment groups and location.

2.8 Histologic and histomorphometric evaluation of the repair tissue

The amount of repair tissue filling the defect was found to be significantly greater in carpal defects (68.41 ± 6.38 %) than in condylar defects (45.85 ± 6.52 %) but was not affected by gene therapy treatment. The composition of the repair tissue with respect to fibrous tissue, fibrocartilage, hyaline-like cartilage and bone was not different between

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treatment groups although there was significantly more hyaline-like cartilage in condylar defects (28.77 ± 4.09 %) than in the carpal ones (5.85 ± 4.01 %).

The histological evaluation of a typical osteochondral section taken through the defect showed that the superficial layer of repair tissue consisted mainly of a thin layer of fibrous tissue with collagen fibers oriented parallel to the surface. Cells present in that layer had a fibroblastic appearance with a fusiform shape and a dense elongated nucleus that had a tangential orientation to the collagen fibers (figure 25). In some sections the fibrous component extended deeper into the repair tissue. The cell morphology remained the same but the collagen fibers seemed thicker and had a more undulating appearance. The middle and deeper layers were composed of a mixture of fibrocartilage, hyaline-like cartilage and bone, with the majority being fibrocartilage (table 4). The fibrocartilage areas were characterized by the presence of dense collagen fibers with a more random orientation than in the superficial layer and, by rounded cells surrounded by a thin layer of pericellular matrix (figure 25). In femoral condyles, the presence of hyaline-like cartilage in the repair tissue could mostly be associated to matrix flow, where matrix from normal cartilage migrated from the edge of the defect into the defect itself forming a lip of hyaline cartilage that served as an anchor for the repair tissue. In radial carpal bones, hyaline-like tissue was usually found in small isolated islands buried in fibrocartilage deep into the repair tissue. The histologic appearance of that tissue was different than that of hyaline cartilage associated to matrix flow seen in stifles. Islands of hyaline-like cartilage were characterized by multiple small clusters of chondrocytes surrounded by pericellular and some interterritorial matrix (figure 26). When seen, collagen fibers were unevenly distributed within the extracellular matrix sometimes making the distinction from fibrocartilage difficult. Extracellular matrix composed most of the hyaline cartilage associated with matrix flow. Chondrocytes were present in small number and usually seen alone or in pairs. Bone was present in one

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third of the defects composing a small portion of the repair tissue in both carpal and condylar defects (table 4). When present, bone was seen at the base of the defects in continuity with the underlying subchondral bone and usually formed a raised platform surrounded by repair tissue (figure 27). The area that would normally correspond to the calcified cartilage layer and tide mark was replaced by a poorly defined region situated between the newly deposited bone or subchondral bone and the fibrocartilage. This region of the repair tissue typically corresponded to an area undergoing bone remodeling and / or osteochondral ossification (figure 28) where both osteoblastic and osteoclastic activities were present. This remodeling area was the same regardless of treatment but represented a greater percentage of repair tissue in carpal defects (table 4). Despite thorough curettage all calcified cartilage was not removed at the time of surgery and some remained at the base of the defect four months later. All but four sections (3 carpal and 1 condylar) showed various amount of remaining calcified cartilage (0.5% to 11.5% of the total defect area). This amount was not significantly different between carpal and condylar defects or between treated, placebo and control defects. The calcified cartilage that remained in the defects was usually found at the edges of the defects in continuity with the one underlying normal cartilage. Its histological appearance was no different than the one of normal calcified cartilage and it was easily discernable from the surrounding repair tissue.

Attachment of the repair tissue to the underlying subchondral bone was calculated by comparing the length of repair tissue attachment to the total length of the defect. Results showed a significantly better attachment to subchondral bone in the carpal defect than in the condylar ones but no treatment effects were noted (table 4). Repair tissue attached to the subchondral bone through the area of active bone and cartilage remodeling mentioned previously. Repair tissue seemed to adhere poorly to remaining calcified cartilage. There was no strong correlation between the length of

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remaining calcified cartilage and the length of attachment of repair tissue to the subchondral bone (linear regression, R2 = 0.37) nor was there one when the percentage of remaining calcified cartilage was used instead of the length (linear regression, R2 = 0.06). However there was a good correlation between the attachment to subchondral bone and the total amount of repair tissue found in the defects (linear regression, R2 = 0.50). Attachment of the repair tissue to normal cartilage was also calculated, comparing the length of the repair tissue attachment to the height of adjacent normal cartilage. The length of repair tissue attachment did not differ between treatment groups and was similar for carpal and condylar defects (table 4). As a general rule most of the repair tissue present at the edge of the defect was attached but it rarely spanned the whole depth of normal cartilage. Unlike attachment to subchondral bone, better attachment to the adjacent cartilage did not correlate with more repair tissue filling the defect (linear regression, R2 = 0.03).

Microfracture puncture sites were seen in some of the sections. They penetrated the subchondral bone to an approximate depth of 3 mm and were usually communicating with the marrow vascular spaces. The tissue filling the puncture sites was intermixed with the one present in the marrow and usually possessed the same characteristics as the repair tissue found above it. Close examination of the subchondral bone at the puncture sites showed evidence of small fissures and sometimes fractures present in the lamellar bone.

The porosity of the subchondral bone underlying the defect (80.36 ± 2.43 %) was similar to the one under adjacent normal cartilage (83. 44 ± 2.34 %) and there was no statistical difference between the porosity of the bone in carpal and condylar defects (83.28 ± 3.36 % vs. 76.95 ± 3.43 %). Furthermore, the various treatment administered to the joints did not affect the porosity of the subchondral bone underneath the defect nor adjacent to it (table 4).

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3.9 Immunohistochemistry evaluation of the repair tissue

Serial sections of repair tissue were stained with antibodies detecting the proteins aggrecan, type I and type II collagens as well as the chondrocyte specific protein S100. Analyzed sections showed different pattern of staining for each protein and, treatment with AdEqIL-1ra and AdEqIGF-1 affected the staining intensity for these proteins.

Type I collagen was detected in all layers of the repair tissue with the same intensity of staining (figure 29); however the staining was more intense in the carpi than in the stifles. Those results were independent of treatments. Type II collagen was also detected in all layers of the repair tissue but this time the staining intensity was greater in the condylar sections than in the carpal ones. The staining pattern was also different than for type I collagen. There was significantly more staining in the deep layer than in all other layers and the superficial layer was significantly less stained than the middle and deep layers (table 5 and figure 30). Gene therapy positively affected staining intensity; sections from treated joints stained significantly more intensely for type II collagen than the placebo and control sections (table 6). Aggrecan staining intensity and pattern were the same as type II collagen with the exception that the staining intensity for both middle and deep layers was the same (table 5 and figure 31). Gene therapy also significantly increased the intensity of aggrecan staining (table 6).

Significantly more repair tissue sections stained positive for S100 in the treated and placebo group than in the control group but the staining pattern was not significantly different between group, that is, the proportion of section that stained positive in the superficial, middle and deep layers of the repair tissue was similar regardless of treatment. Staining patterns for S100 were also similar for carpi and stifles section.

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All negative control sections co-incubated with anti-serum or specific antigen to the antibody did not show any trace of staining for the previously mentioned proteins.

3.10 Glycoaminoglycan and water content of the repair tissue and adjacent normal cartilage

3.10.1 Adjacent normal articular cartilage

The dimethylmethylene blue assay performed on normal cartilage adjacent to full thickness articular cartilage defects showed that there was significantly more GAGs in the normal cartilage of femoral condyles (mean ± SE = 211.23 ± 9.01 µg/mg dry weight (dw)) than in the normal cartilage of radial carpal bones (mean ± SE = 176.45 ± 9.01 µg/mg dw).

Water content in the areas adjacent to defects was also calculated and analyzed. Results show that articular cartilage of medial femoral condyles adjacent to chondral defects contain significantly more water (74.6 ± 0.77%) than normal cartilage adjacent to defects created in radial carpal bones (66.66 ± 0.77 %).

The injection of AdEqIL-1ra and AdEqIGF-1 or the placebo did not affect the amount of GAGs or the percentage of water present in normal cartilage adjacent to both stifles and carpi defects (no treatment effect).

3.10.2 Repair tissue

Analysis of the GAG content of the repair tissue showed a distinct treatment effect. The GAG content of repair tissue present in treated joints and placebo joints was significantly higher than the GAG content in repair tissue coming from control joints (Figure 32). Intra-articular injection of AdEqIL-1ra and AdEqIGF-1 increased the amount of GAGs in repair tissue by 30.86% when compared to control joints of horses that received Gey's balanced salt solution but were not exposed to the viral preparations.

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Placebo joints that were also injected with Gey's balanced salt solution but were the contralateral joints of treated joints showed a 45.75% increased in GAG content when compared to control joints. This increase was not significantly different from the one seem in the treated joints (figure 32). Again, the amount of GAG present in the repair tissue of defects of femoral condyles (112.26 ± 10.45 µg/mg dw) was significantly greater than the amount present in the repair tissue found in carpal defects (50.76 ± 10.45 µg/mg dw). On average, when compared to normal adjacent cartilage, repair tissue from femoral condyles regained up to 60% of their initial GAG content (60.83 ± 6.06%), while repair tissue from radial carpal bone defects regained significantly less (30.56 ± 5.93%). However, this time, the percentage of water contained in the repair tissue at both location was not significantly different (stifle: 78.18 ± 1.45 %, carpi: 76.73 ± %) Even if the treatment with AdEqIL-1ra and AdEqIGF-1 increased the amount of GAGs present in the repair tissue, it did not affect the percentage of water present in the tissue after four months of healing. When compared, the percentage of water in the repair tissue (77.24 ± 0.79%) was not statistically different from the one seen in the normal adjacent cartilage (70.64 ± 0.92%).

3.11 TaqMan real time PCR evaluation

Messenger RNA expression for various proteins implicated or with potential implication in cartilage healing was evaluated in synovium, repair tissue and normal cartilage adjacent to the defects collected at necropsy 16 weeks post-operatively. Message for IGF-1 was detected in all tissue types. Expression levels in synovium and cartilage adjacent to the defect were similar and were not affected by treatments. However, mRNA expression varied with treatment in the repair tissue. The level of expression was higher in repair tissue from control joints than from repair tissue from placebo joints and, although the level of expression was higher in control joints than in

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treated ones, the difference was not statistically significant (figure 33). IGF-1 mRNA expression was significantly higher in the cartilage adjacent to the defect than in the repair tissue in both the treated and placebo joints but, IGF-1 expression was the same for both type of tissues in the control joints (figure 33).

Synovial membrane IL-1ra mRNA expression was similar in all groups. It was also similar to the expression seen in the adjacent cartilage and repair tissue from the joints of the control group, but was significantly higher than the IL-1ra expression in the adjacent cartilage and repair tissue of the treated and placebo joints. There was no significant difference between the IL-1ra expression levels in the repair tissue and the cartilage adjacent to the defects; however expression levels in both tissues were higher in the control joints than in treated and placebo joints (figure 34). There was no significant difference in the mRNA expression of treated and placebo joints in both groups.

Type I and type II collagen mRNA expressions in synovial membrane, repair tissue and cartilage adjacent to the defect were not affected by treatment. On the other hand, type I collagen expression seen to be higher in cartilage adjacent to the defect than in repair tissue while type II collagen mRNA expression was similar in the two tissue types (figure 35, 36). Type I collagen expression was high in synovium while type II collagen was low. As for type I and type II collagens, levels of aggrecan mRNA were similar in all treatment groups, and as type II collagen, the aggrecan expression was the same for repair tissue and cartilage adjacent to the defect (figure 37).
Messenger RNA expression of the two cytokines, IL-1α and TNFα, was not significantly different between control, treated and placebo joints. TNFα expression levels were significantly higher in the repair tissue than in the cartilage adjacent to the defect, but this increased expression was not seen for IL-1α mRNA. Synovial membrane had the lowest IL-1α mRNA expression while synovial membrane TNFα mRNA was higher than

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cartilage adjacent to the defect but not than repair tissue (figure 38, 39). Similar results were seen for the mRNA expression of metalloproteinases MMP3 and MMP13, their level of expression was not affected by treatment; they both had significant higher expression in repair tissue than in adjacent cartilage. For MMP3, synovial membrane expressed slightly more mRNA than adjacent cartilage but not enough to make it significant but enough to make it comparable to the level of expression seen in the repair tissue. For MMP13 synovial membrane expression was similar to the one of cartilage adjacent to the defect, therefore significantly less than in the repair tissue (figure 40, 41).

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CHAPTER 4

4 Discussion

Healing of full thickness chondral defects has been the focus of multiple research projects. In results of these projects, various surgical techniques were developed and used clinically to improve cartilage healing and, beneficial factors to cartilage healing were identified and tested in *in vivo* models of chondral defects. A new era has now begun where researchers combined surgical techniques with the use of these factors in an effort to further improve the quality of repair tissue seen in healing chondral defects. So far most of these projects have involved implanting a matrix containing the molecules of interest in the defects and evaluating the quality of repair after various periods of time. [354, 355, 178, 242, 243] A small numbers of projects have looked at gene therapy as a potential delivery method for those proteins, but again it was based on matrix implantation to deliver ex vivo genetically modified cells to the defect. [244, 345, 346, 347] As mentioned in the introduction this method requires two surgeries, one for cell collection and one for reimplantation, as well as an extended period of in vitro culture to infect and select the transduced cells. All these extra steps add to the cost of the procedure as well as to patient morbidity.

The study presented here, used an equine model and combined the use of SBPM, a technique proven effective in enhancing cartilage healing in that species, [182] with the IA injection of adenoviral vectors carrying the genes of equine IL-1ra and IGF-1. These viral vectors were engineered to safely infect cells in vivo and produce the two proteins shown to have positive effect on cartilage healing, [242, 243, 277] eliminating

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the need for tissue or cell-seeded implants and additional surgeries. This project also paid particular attention to the composition of the repair tissue, as it has been shown that the incapacity to significantly increase the concentration of proteoglycans in the repair tissue has been one of the major problem in all the techniques evaluated so far. [182, 356]

Full thickness chondral defect created in the distal surface of the radial carpal bone and in the medial femoral condyle of the horse were chosen as the experimental model for naturally occurring lesion to the cartilage. Numerous reasons led to the selection of this particular model. The equine model shows major advantages over the more widely used rabbit and dog models. In human, cartilage damage occurs most often in areas of maximal weight bearing, the femoral condyle being one of the most frequently affected. [357] The equine stifle is the equivalent of the human knee and the cartilage in the weight bearing area of the equine femoral condyle is similar in thickness to that of the human (2 to 3 mm) compared to the dog (0.5 to 0.8) [358] and the rabbit (0.2 to 0.3 mm). [359] Furthermore, the biomechanical forces applied to the equine femoral condyle during weight bearing are presumably equal or greater than those experienced by the articular surface of the human femoral condyle. [182] The distal surface of the radial carpal bone is the most commonly reported site affected by osteochondral fractures and associated degenerative lesions in racing horses. [185] In human osteoarthritis, the damage to articular cartilage rarely extends past the subchondral bone plate. Virtually all the reported studies investigating cartilage repair using animal models have involved the creation of full thickness defects extending deep into the subchondral bone initiating a spontaneous repair response irrespective of the treatment applied [360], whereas in our model the surgical technique has been developed to remove all the calcified cartilage but preserve the subchondral bone plate. Horses can easily be trained to run on a treadmill allowing the elaboration of a controlled

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exercised program that can be compared to the one human patients are put through following surgery. Equine athletes naturally sustain articular cartilage lesions at similar anatomic locations to the experimentally created defects [183], allowing us to predict more accurately the healing process and clinical response to treatment of naturally occurring lesions that could potentially be treated with the new technique. The decision to use both carpal and femoral locations was, as mentioned previously, linked to the natural occurrence of the lesions in these locations but also to the ease of carpal arthrocentesis and the historical better healing in femoro-condylar defects. [182] All these factors makes our equine model a more rigorous model to evaluate the effectiveness of repair enhancing techniques and gives more weight to the positive results obtained using the combined SBPM – gene therapy technique.

All the surgeries were performed without major problems except for the creation of an osteochondral fragment on the radial carpal bone in 3 joints. These small fragments were all removed at the time of surgery and did not seem to have caused any problems since the sites they originated from were barely visible at the time of gross evaluation 16 weeks later and, did not affect the grading of the repair tissue.

The measure of IL-1ra concentration in the synovial fluid of the middle carpal joints confirmed that AdEqIL-1ra / AdEqIGF-1 treated joints produced significantly more IL-1ra than the joints that were not treated. These results indicate that the AdEqIL-ra vector was able to infect the cells of the joint and use the cells transcription / translation machinery to produce and secrete the protein encoded by the IL-1ra transgene. IL-1ra protein concentrations remained significantly higher in the treated group for a period of 21 days indicating that the viral vector remained present and active in the infected cells for at least that period of time. The length of expression and concentration of IL-1ra compares favorably to the ones obtained in a study that looked at the use of IL-1ra gene transfer for the treatment of osteoarthritis in an equine model. [280] The production of

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viral proteins in adenovirus transduced cells and their elimination by the cellular immune response mediated by cytotoxic T lymphocytes is probably the reason behind the decreased IL-1ra levels associated with transgene expression. [361]

An increase in IL-1ra concentrations were seen 1 week post treatment in the contralateral placebo treated carpi. The increase only persisted for one week but was significantly higher than the baseline concentrations seem prior to defect creation and treatment. Since that increase was not noticed in the carpi of horses that were never exposed to the viral vector (control joints), it is possible to speculate that systemic levels of IL-1ra were attained during the peak intra-articular expression of IL-1ra in the treated joints, and this was seen 1 week post injection. Peak IL-1ra concentrations reached levels as high as 540 ng/ml, and it is possible that with distribution it reached levels higher than the 0.02 ng/ml baseline concentration in the contralateral joint. Systemic drug concentration following intra-articular medication is well documented in the horse [362-364] but concomitant levels in non-treated joints has not been reported. On the other hand, a gene transfer study using a rat adjuvant arthritis model, showed increased level of the transgene protein IL-4 in plasma and in the contralateral paw 8 days after IA injection of a retroviral vector. [365] Since we did not measure IL-1 concentration in blood and in intact joints during our study, this explanation remains purely hypothetical. A similar phenomenon was reported in an equine study using AdEqIL-1ra in intact carpi, but the increase in IL-1ra in the contralateral limb was not seen until day 28 after transfection where transgene expression had most likely ceased and was paralleled by an increase in the treated joints suggesting an endogenous response. [366] Another potential explanation involves the trafficking of genetically modified dendritic cells and macrophages to distant inflammation sites. This phenomenon has been well documented in models of immune mediated arthritis in rabbits and mice. [367, 368, 328, 196] However, in these cases, increase in the concentration of the transgene protein

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was not seen in the contralateral joints but, their synovial fluid was diluted by joint lavage and, therefore detection of low levels of protein might have been difficult. Then again, it is possible that IL-1ra synthesis by trafficking cells was not the only cause of increased IL-1ra levels in the contralateral joint in our study. Up-regulated synthesis by resident cells mediated by the dendritic cells could be another possible explanation. [368]

All joints, including controls, showed a significant increase in synovial fluid IGF-1 concentrations following surgery and treatment and, the elevated levels persisted for at least 6 weeks post-treatment. However, unlike IL-1ra, higher concentrations of IGF-1 were not detected in the joints that were injected with the viral vectors. It is always possible that in vivo transduction with the AdEqIGF-1 did not work. However in vitro testing of the viral preparation prior to its use in the horses showed a significant increase in IGF-1 concentration in treated equine synoviocytes when compared to controls (data not shown). A harmful interaction between the two viral vectors affecting the transfection of one but not the other is unlikely since in vitro work was successful in transducing equine synoviocytes with both vectors. [369] As mentioned previously, joints of horses that were never in contact with the viral preparations showed a significant increase in their IGF-1 concentration. These results suggest that factors present or released during cartilage healing up-regulated the expression of endogenous IGF-1 for a prolonged period of time. This increased endogenous production could potentially mask a low concentration of protein synthesized by the transgene. To minimize the immune response associated to high doses of two viral vectors, the number of AdEqIGF-1 particles injected in the treated joints ($10X10^{10}$) was less than the number necessary to obtain optimal protein production [Nixon AJ, personal communication] Therefore it is plausible that only a low concentration of IGF-1 was obtained and masked by the endogenous response. Endogenous increase in synovial fluid IGF-1 levels in joints with healing chondral defects has not been documented until now. However one study,

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performed in cartilage defects created in equine femoral trochlea, reported IGF-1 mRNA expression in healing tissue that parallel the synovial fluid concentration increase seen in our model. [186] Another study, using the rabbit ear as a model, demonstrated peak expression of IGF-1 protein in healing tissue seven days after defect creation using an immunohistochemistry technique. [370] These increases are not surprising since IGF-1 is known to play major roles in cartilage anabolic metabolism as well as in chondrocyte proliferation and differentiation. [196-198] Factors present in the healing joint environment that may up-regulate IGF-1 expression have not been studied but, growth hormone, low concentration of PGE_2 and IGF-1 itself, through an autoinductive autocrine/paracrine response, have been shown to increase IGF-1 mRNA expression in chondrocytes and differentiating mesenchymal cells of the growth plate. [234-237] Another potential element responsible for the early increase in IGF-1 would be the release of IGF-1 from IGFBP-3/fibronectin complexes from stores present in the cartilage extracellular matrix following cartilage injury. [371]

Clinical examinations of the horses indicated that, although severity of lameness increased from week 0 to week 16 and had a tendency to be more severe in the forelimbs, increased expression of IL-1ra and IGF-1 did not appear to affect the degree of lameness seen in the horses following surgery. The amount of joint effusion was also not affected by treatment with AdEqIL-1ra and AdEqIGF-1. These results differ from those of another study were gene transfer of IL-1ra decreased both lameness and joint effusion scores in a equine model of OA, [280] but is in agreement with a previous SBPM study where, 4 months after defect creation control and SBPM treated joints did not show differences in degree of lameness. [182] The absence of differences between groups could be attributed to the mild lameness and synovial effusion associated with cartilage defects, the presence of defects in 4 limbs and the subjectivity of lameness evaluation and effusion scoring or to the fact that there were simply no differences.

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Therefore more severe lameness and joint effusion seen in the equine osteochondral fragment model [280] and the fact that lesions were only created in carpi probably explains the different results between our study and the OA/gene therapy study. In the future, to avoid missing possible mild treatment effects, measure of the joint circumference and the use of force plate analysis for lameness evaluation might be implemented. [278, 372] Although force plate analysis allows us to identify the lame limb it gives no information on the location of the pain. Therefore unless we use local anesthesia we cannot confirm that the observed lameness is associated to the presence of the defect.

Even if synovial effusion was similar between all treatment groups, statistical analysis of the results showed a significant difference between carpi and stifles as well as between weeks into the project. The lower scores given to the stifle joints may be related to the presence of larger muscle masses in the area of the joint, making evaluation of mild synovitis more difficult and less precise. It may also have to do with the fact that the same amount of viral particles was injected into two joints of different volume creating less inflammation in the larger femorotibial joint. The decrease in carpal effusion seen during the second half of the study may partly be explained by the decrease in the frequency of arthrocentesis but, since the same decrease was seen in the untapped stifles some physiologic event taking place in all joints was probably involved. From the results of synovial fluid analysis it is safe to assume that it is probably associated to the return to baseline levels of all the inflammatory parameters. Using carpal defects as our experimental model allowed the weekly collection of synovial fluid from the healing joints and, characterize the joint physiologic response to the creation of full thickness chondral defect as well as to monitor the effect of the injection of AdEqIL-1ra and AdEqIGF-1 in to those joints. Analysis of the synovial fluid showed that most parameters routinely measured to assess articular inflammation were elevated

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in all groups following defect creation and treatment and, were more pronounced and persisted for a longer period of time in joints that received IA gene therapy. The increase in total protein, decrease in the quality of the mucin clot, and absence of cellular response in synovial fluid early after the creation of chondral defects has been reported previously, [183, 242] but an increase in PGE_2 concentration has not. It is likely that the trauma of surgery and defect creation are responsible for these changes. Although arthroscopic techniques are less invasive and less traumatic to tissues than arthrotomies, they still damage the synovium through repetitive penetrations of instruments through the portals and continuous lavage. [373] Furthermore, small cartilage debris left over after surgery, despite thorough lavage, can perpetuate the inflammatory response. [374] Early after defect creation damaged cartilage at the edges of the defect and the exposed subchondral bone most likely release inflammatory mediators and pro-inflammatory cytokines in the joint environment contributing to the inflammatory reaction and explaining the increase in PGE_2 and total protein. Interestingly enough this reaction takes place without a cellular infiltration, indicating that the resident cells of the joint are the sole responsible for this reaction. Most of the inflammatory parameters were back to baseline values within 2 weeks of defect creation in the control joints but, surprisingly PGE_2 levels remained elevated for up to week 5. Prostaglandin E_2 is mostly known for their inflammatory properties however, numerous reports have shown that it can also have anabolic effects on chondrocytes. Depending on the physiologic conditions it can have simulatory effects on cell proliferation, differentiation and matrix synthesis. [375-377] Furthermore they probably mediate the some of the anabolic effects of TGFβ and acts as a secretagogue of IGF-1. [378, 379]

As mentioned previously the inflammatory reaction seen in the treated joints differed from the response seen in the control joints. Joints treated with the adenoviral vectors had a significant cellular component to the inflammatory reaction characterized

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by an early increase in the total WBC followed by a chronic elevation in the numbers of lymphocytes present in the synovial fluid. Lymphocytic infiltration in tissues following gene transfer using modified adenoviral vectors is well documented. [380-383] A particular study has characterized the inflammatory response of the synovial membrane to IA adenoviral vector injection and has found sequences of events similar to ours. [383] An early, predominantly neutrophilic inflammation in the synovial tissue was first seen, followed on day 7 by the peak inflammatory response consisting of a cell population comprising mostly of neutrophils and monocytes. By day 21 the inflammation had significantly decreased and was primarily a lymphocytic infiltration. The persisting lymphocytic infiltration as well as the chronic increase of the synovial fluid lymphocytes can probably be associated to the B-lymphocyte population responsible for the production of antibody against the viral proteins. [384] The cellular response seen in our study differs slightly from the one seen in intact carpi and carpi with an osteochondral fragment where a similar adenoviral construct was used. [366] Intact carpi transduced with the adenoviral vector did not show any increase in the lymphocyte count while carpi with osteochondral fragment demonstrated a rise in mononuclear cells before an increase in lymphocyte count. The differences between the two studies could be explained by the differences in the models as well as differences in viral preparation potency, purity and concentration. [385]

As in the placebo and control joints, an increase in PGE_2 concentration was seen in the treated joints following defect creation; however, this increase was significantly greater than in the two other group of joints and persisted for a longer period of time. Multiple in vitro studies performed on synoviocytes and chondrocytes have shown the potential of IL-1ra to decrease the PGE_2 production induced by IL-1. [280, 386-387] Furthermore in vitro experiments conducted to test the efficacy of the viral preparation before its use in vivo showed that the peak concentrations measured in vivo were

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comparable to in vitro concentrations capable of inhibiting the induced PGE_2 production in cultured synoviocytes stimulated with 10 ng/ml of IL-1 (data not shown). This suggests that the beneficial effects of IL-1ra on cartilage seen in vivo may be mediated by a mechanism independent of the inhibition of IL-1 induced PGE_2 production. The binding of IL-1 to its specific receptor may decrease the IL-1 mediated metalloproteinase production by chondrocytes, [388] as well as down regulate the synthesis of chemokines by activated synoviocytes therefore, reducing the migration of inflammatory cells to the joint. [389] Reasons for the persisting high concentrations of PGE2 despite the expression of therapeutic levels of IL-1ra could be associated with the presence of other cytokines capable of inducing its production. [390, 391] However this does not explain the greater increase in PGE_2 seen in the treated joints when compared to the control and placebo joints nor the prolonged increase in total protein and lymphocyte count. In order to get therapeutic levels of both IL-1ra and IGF-1 we were forced to use a combined viral concentration greater than the ones shown not to elicit an inflammatory response in healthy carpal joints ($< 20X10^{10}$ particles). [280] Even though there is no previous data confirming that $30X10^{10}$ viral particles will initiate an inflammatory response in equine carpi, historical data has shown that a viral load of $50X10^{10}$ particles was associated with a significant increase in the total WBC count in those joints. [280] Therefore it is possible that by increasing our viral concentration by a factor of 0.5 we have reached the threshold viral load where a significant inflammatory reaction is induced, explaining the previously mentioned changes. It is also possible that the changes in the joint environment, initiated by the creation of the defect, sensitized or activated cells in the joint tissues making them more responsive to a lower viral load. It is interesting to note that although the inflammatory parameters values measured in the placebo joints were similar to those of the control joints, they took significantly longer to return to baseline levels. This finding could solidify our hypothesis that transduced dendritic cells migrated

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to the contralateral carpi inducing IL-1ra production and increasing the length of the mild inflammatory response.

Some radiographic changes were present in both carpi and stifles 16 weeks after defect creation. The observed changes were mild and seen only in a small number of joints indicating that the presence of full thickness chondral defects is not associated with the rapid onset of severe osteoarthritis like the carpal osteochondral chip model that has been used as a model of OA in our laboratory. [280] Although changes in the radiographic appearance of the subchondral bone were seen there was no difference between location or treatment; furthermore, formation of complete subchondral cysts was not apparent in this study while others have reported their formation in femoral condyles and in radial carpal bone following creation of full thickness chondral defects with removal of the calcified cartilage layer. [182, 184] The different findings may lie in the different length of the studies. Our experimental period was 16 weeks while the other two studies extended up to one year after defect creation. It is possible that over time the mild changes seen in our horses would have progressed to more severe lesions and become comparable to what has been described in other studies. Osteophyte formation was significantly more severe in placebo carpi than in treated carpi and although osteophyte formation was more severe in the control carpi than in treated carpi, the difference was not significant. There was no difference in osteophyte formation in the stifle joints. The difference in lesions severity between carpal groups may be associated to the anti-inflammatory properties and protective effects of IL-1ra. IL-1ra has been shown to decrease the manifestation of osteoarthritis in an equine model [280] and, significantly reduced the radiographic progression of joint disease in a clinical study of patients with rheumatoid arthritis. [275] The absence of differences in osteophyte formation between the treated and control groups may be related to the low incidence of radiographic changes, the low grade of the changes and the small number of joints in

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each group. The absence of difference between the treatment groups in the stifle is harder to explain especially since there was a significant difference in the carpi. The medial femorotibial joint is larger than the middle carpal joint and it communicates with the femoropatellar joint increasing the volume of distribution of the viral preparation. Therefore it is possible that the concentration of IL-1ra that was reached in the stifle joints was not high enough to protect against osteophyte formation but sufficient to positively affect the quality of the repair tissue.

The defects were not completely healed at 16 weeks but were filled to various degrees with repair tissue that was white to transparent in color and presented some degree of surface irregularities. Although some significant differences were noted between the healing of carpal and femoral condylar defects, gross distinction between the healing of gene therapy joints and control or placebo joint was impossible. Historic data has shown that healing of large equine chondral and osteochondral defects was not complete even one year after defect creation. Furthermore, for most studies, there was no further improvement in the quality and quantity of the repair tissue past 4 to 6 months of healing, [177, 182, 184, 355] justifying the length of our experimental period.

The disparity between the healing of joints of the same group in different horses and even between joints of the same group in the same horse is a common occurrence [177, 182, 184, 356] and is part of the intra-species variation that needs to be considered in the experimental design. So far no good reason has been able to explain these occurrences. Differences in nutritional state or difference in exercise levels cannot account for such variation in healing since those variables are controlled. On the other hand metabolic state, horse temperament affecting activity levels or even, differences in the progression of the healing process between breeds cannot be controlled and are potential factors implicated in the wide range of healing responses seen in these studies. However these factors still do not explain wide intra-horse variations. The presence of

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mainly two types of repair tissue has been reported in many studies involving equine cartilage healing. [177, 182, 183 ,392, 393] The dense white colored tissue has often been identified as fibrocartilage or immature hyaline cartilage while the translucent tissue has been associated to fibrous tissue. [392, 393] Another study relates the gross appearance of the repair tissue to its thickness rather than to the presence of fibrocartilage. [183] The observations made in this study would support the latter observation.

The absence of macroscopic differences, except for repair tissue firmness, between treated, placebo and control group is disappointing but not entirely surprising considering the absence of differences in IGF-1 production between the three groups. Because of its anabolic properties and its effect on cells proliferation and differentiation IGF-1 was the factor that we thought would have the most effect on enhancing the quality or at least the quantity of the repair tissue. Although IL-1ra was used in this study it was mostly selected for its anti-inflammatory properties and was expected to complement or accentuate the effects of IGF-1 more than to initiate a significant healing response. The short length of expression of the IL-1ra transgene compared to the relative long term of the project may also explain the lack of difference in the gross appearance of the repair tissue between groups. It is possible that the anti-inflammatory properties of IL-1ra combined to the endogenous effects of IGF-1 and potentially the effects of other growth factors present in the joint caused early benefic changes that could no longer be observed 16 weeks post-defect creation. We also need to consider that the peak inflammatory reaction was present at the time of peak IL-1ra production and that some of the beneficial effect of IL-1ra might have been nullified by the concomitant inflammatory response. Firmness of the repair tissue was the parameter that was statistically different between groups, with tissue from the placebo group being firmer than the one in the control and treated groups. It is difficult to attach a lot of

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importance to this result considering the subjectivity of the measuring technique. The use of a tissue forceps to indent the repair tissue is extremely imprecise; the use of an intender would have been preferable and biomechanical testing would have been ideal. [394, 395]

In this study macroscopic evaluation has shown that the volume of the repair tissue present in the carpal defect seemed to be greater than in the femoral condylar defects. This finding is interesting since a previous project using the same model showed that condylar defects tended to heal better than carpal ones. [182] Two different surgeons created the carpal and condylar defects allowing for possible difference in the removal of the calcified cartilage layer and the way to perform the SBPM technique which are two factors that could have had an effect on the volume of repair tissue present in the defect. [182, unpublished data] However histomorphometric analysis showed that the amount of calcified cartilage leftover after surgery was the same in both carpi and stifles. On the other hand, the number of perforations created in the femoral condyles was greater than in the radial carpal bone for a defect of approximately the same size but, the microfracture holes seemed to penetrate deeper into the subchondral bone in the carpal defects. At this point, it is impossible to confirm that these factors had anything to do with the difference of healing seen in the two locations. Differences in carpal and femoral condylar defects also extended to the healing pattern of the defects. Condylar defects heal with a considerable amount of matrix flow while carpal defect have a tendency to heal from the center with minimal contribution from the surrounding normal cartilage. These different healing patterns have been documented before. [177, 396 ,168, 169] One study even credits matrix flow to be responsible for 50 to 90% of the healing of small femoral trochlear lesions (≤ 5mm) and 36% of 15 mm lesions. It also associates the difference between carpal and femoral healing to the difference in cartilage thickness in these areas. [168]

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Four months following defect creation some mild changes were still present in the synovial membrane. The mild intimal hyperplasia and subintimal fibrosis are probably secondary to surgery and the continuous presence of the defect causing a mild chronic synovitis [242] and, is probably not associated with the use of the adenoviral vectors since the changes are the same in all groups. However the lymphocytic infiltration is most likely caused by the vectors. [383, 397] The lack of difference between the treated joints and the control and placebo joints, even though the infiltration was more severe in the treated joints, can be explained by the gradual decrease in the inflammatory response following elimination of the infected cells. Synovium samples taken earlier in the study might have shown more significant changes.

The lymphocytic infiltration in our model is more persistent than any described so far. A study in a mouse reported a resolution of the infiltration 3 weeks following IA administration of the vector which seems extremely short considering that the expression of the transgene was at least 14 days. [383] However they were using joints exempt of pathology which could explain the brief inflammation. One other study, in a dog model of osteoarthritis, reported persisting marked cellular infiltration 4 weeks after vector administration [397] and, a similar study conducted in the horse showed lymphocytes in the synovial membrane subintima 54 days post vector injection. [280] One factor that might have contributed to a more severe infiltration in the treated joints of our model, is the higher initial viral load injected into the joints. We already have determined that it was probably responsible for the high lymphocyte count present in the synovial fluid and, since these changes persisted for 8 weeks it is logical to assume that it could cause a cellular infiltration in the synovial membrane for as long as 16 weeks post treatment.

Histomorphometric evaluation of osteochondral sections of the defects and repair tissue confirmed the results obtained from macroscopic evaluation. The amount of

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repair tissue present in the defect was the same for all treatment groups and carpi had more of it than stifles. Histomorphometry also showed that the percentage of each type of tissue present in the defect was not different between treatment groups but differed between carpi and stifle. Two microfracture projects performed in horses and one performed in dogs showed similar findings. [181, 182,356] It has been proposed that the microfracture technique enhances cartilage formation by providing better access to the growth factors and undifferentiated mesenchymal cell population present in the bone marrow. In both equine studies care was taken to remove the calcified cartilage layer providing access to the bone marrow. In the dog study, when microfracture was not performed, an exogenous source of chondrocytes was placed in the defect. This similarity between studies seem to indicate that facilitating cell migration and possibly cell proliferation is sufficient to increase the amount of repair tissue present in the defect but insufficient to induce production of hyaline cartilage. None of these studies tried to modulate cell differentiation which appears, according to these results, to be one of the key step in achieving a repair tissue with more hyaline cartilage.

An interesting series of studies performed in adult mini pigs with partial thickness defect reinforces that point. [398, 194] Synovial cells were able to migrate and proliferate in partial thickness defect when a space-filling matrix containing the chemotactic/mitotic TGF-β1 was introduced into the defect. However the repair tissue that resulted from this experiment was a primitive type of scar tissue consisting mainly of fibrous tissue that failed to transform into cartilage. When a time-release-liposome-encapsulated chondrogenic factor (high concentration of TGB-β1) was introduced in the matrix the defects became filled with cartilage like tissue containing all the major cartilage matrix components. An important point stressed by the authors of this study was that the timing of the release of the chondrogenic factor is of critical importance. It needs to be introduced when cells have already migrated and proliferated so the growth

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factor can have its maximal effect. The choice of the growth factor is also primordial as well as its concentration, the method of introduction to the defect and the environment to which it is introduced. The same group that had very successful results using TGF-β1 in a partial thickness defect model was left with a defect filled with bone when they used the same delivery system for TGF-β1 in full thickness articular cartilage defects. [399] Other studies have tried to modulate cell differentiation by the addition of growth factors to the defects. [178 ,194, 242, 354, 355, 398 ,400, 401] Results varied depending on the model, the growth factor utilized and the methodology used to identify the presence of hyaline tissue but, in general, it appears that there was more tissue with hyaline like quality in the defects of those projects. Our own study tried to enhanced the quality and quantity of the repair tissue but still failed to show an increase in the amount of hyaline cartilage seem in the defect although tissue with greater GAG content was seen. Beside our incapacity to increase the level of IGF-1 above endogenous level, the choice of the growth factor itself might be implicated in these disappointing results. It has been shown that IA injection of IGF-1 into mice knees did not consistently stimulate the production of proteoglycans while growth factors from the TGFβ superfamily did. [HHH] Furthermore, another study classified IGF-1 as a protein more involved in cartilage maintenance than cartilage repair. [398]

Hyaline cartilage comprised a greater proportion of the repair tissue in stifles than in carpi. Histologic evaluation of the healing pattern in both locations makes it easy to understand that extensive matrix flow associated with cartilage healing in the stifle is responsible for this difference. As mentioned previously it has been reported that intrinsic healing by matrix flow can by responsible for up to 36% of the repair tissue present in femoral defects. [168] Although the proportion of repair tissue originating from matrix flow has not been measured in our study, we can approximate a value of 23%

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assuming that the proportion of hyaline cartilage not associated with matrix flow is the same in carpi and stifles.

As with increasing the proportion of hyaline cartilage in the repair tissue, better attachment of that repair tissue to subchondral bone has been a source of problem with many techniques. So far, it seems accepted that removing the calcified cartilage layer is one way to achieve acceptable attachment of the repair tissue to the subchondral bone, [177, 182] although some groups still believe that intact calcified cartilage is essential for bonding between repair tissue and the base of the defect. [356] In our study, even though there was no difference in the percentage of intact calcified cartilage between the carpi and stifles, significantly better attachment of the repair tissue to the subchondral bone was seen in the carpal defects. However, after closer evaluation of the results it was demonstrated that carpal defect had a greater area of remodeled tissue at the base of the defect where the calcified cartilage layer used to be and; it seem that better attachment might have been present in those defects. Unfortunately no correlations were found between the presence of the remodeling layer and attachment of the repair tissue to the subchondral bone (results not shown). Subjective observations made during surgery seem to suggest that microfracture perforations made in the carpal defects were deeper than the ones in the femoral condyle. It is possible that the depth of the microfracture carries more importance in the attachment of the repair tissue to the underlying bone than the number of perforations present in the defects. Deeper perforations might allow better anchorage for the repair tissue than more shallow ones. Breinan et al. using the dog as a model have mentioned that removal of the calcified cartilage layer and exposure of the subchondral bone is associated with more repair tissue filling the defect but also with less hyaline-like tissue. [356] However they also reported that exposed subchondral bone may be important to the bonding of the repair tissue to the base of the defect by allowing the incorporation of the newly synthesized

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collagen fibers followed by their anchoring to the subchondral bone by remineralization of the matrix. [195] On the other end Hanie et al., working with horses, suggest that the subchondral bone is the source of hyaline cartilage and healing of cartilage defect may be improved by penetrating the subchondral bone plate. [177] Previous work using our microfracture model has shown that improving access to the subchondral bone does indeed increase the amount of repair tissue in the defect but has failed to show improvement in the amount of hyaline cartilage. [181, 182] It also showed that removal of the calcified cartilage correlated with better attachment of the repair tissue (unpublished data). To complement these findings the present project demonstrated that better attachment to the subchondral bone was correlated to an increase in the amount of the repair tissue. The results of these studies indicate that cartilage healing is affected differently by the presence or absence of the calcified cartilage layer, therefore, before we can make a final decision as whether it should be removed or not, more work has to be done on its effects on the healing process.

The use of AdEqIL-1ra and AdEqIGF-1 did not stimulate the reformation of the tide mark or calcified cartilage layer. The layer of remodeling/mineralizing tissue replacing the original calcified cartilage layer was similar between all treatment groups and locations. The recreation of the original barrier between the subchondral bone and articular cartilage has been documented in very few studies and even then the authors' description of the tide mark is not clear enough to determine whether or not they are describing the area of remodeling/mineralizing tissue mentioned previously. [355] If indeed they are talking about a well organized structure possessing the characteristics of the tide mark then, the use of BMP-2 seems to help in its reformation. [355, 401]

Incomplete integration of the repair tissue to the surrounding normal cartilage has been described as one of the reason associated with repair failure in numerous techniques employed to promote cartilage healing. [295, 402-404] The subchondral

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bone plate microfracture technique is not different from the others and gene transfer using the IL-1ra and IGF-1 genes did to affect that parameter of healing. In most of the defects, repair tissue tended to attached by a small sliver of fibrous tissue to the adjacent normal cartilage. Numerous reasons have been given to explain the poor integration of repair tissue to its adjacent cartilage. Lack of matrix producing cells in the cartilage-cartilage interface area due to chondrocyte death at the lesion edges, avascularity, low number of pluripotent progenitor cells and poor chondrocyte migration through the dense extracellular matrix have all been deemed contributing factors. [405-407] Other authors suggest that impaired integration can also be related to the anti-adhesive properties of proteoglycans such as decorin, biglycan and aggrecan. It appears that through an interaction dependent on their glycoaminoglycan components, they are able to block adhesion to molecules like collagen and the cell binding domain of fibronectin. [408-410] Digestion of the cartilage extracellular matrix with chondroitinase ABC and trypsin has therefore been suggested after debridement of lesions and before implantation of constructs or cells. [172] The in vitro use of collagenase has also been explored and results showed an increase in chondrocyte density at the wound edges and better integration of the cartilage implant. These positive results have been attributed to cell migration and proliferation induced by the disruption of the collagen architecture. [411] If the major obstacle to integrative repair is indeed the inhibition of adhesion, it is not surprising that anti-inflammatory agents and growth factors do no seem to enhance integration of the repair tissue to cartilage. However it might benefit cell migration and proliferation following collagenase treatment.

Even though microscopic evaluation failed to show an increase in the amount of hyaline like tissue in the treated defects, biochemical analysis was able to determine that IA gene transfer increased the amount of proteoglycans (PGs) present in the repair tissue of the treated defects as well as in the contralateral placebo treated ones. The

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mechanisms involved in this positive effect of the combined SBMF / gene transfer treatment on proteoglycans metabolism are not clear. Results have shown that IGF-1 expression was not increased above the normal physiologic response to defect creation in the treated joints and, that the high concentration of viral particles induced an acute inflammatory response and chronic elevation of the lymphocyte count in the synovial fluid of those joints that was not present in the placebo treated and control joints. It also showed a mild and transient increase in the IL-1ra levels in the placebo joint where as no such increase was seen it the control joints. Therefore it is unlikely that the growth enhancing properties of IGF-1 were the sole responsible for the increase in PGs otherwise we would have seen a similar response in the repair tissue of the control joints. Other mechanisms and factors most have come in to play. Since the only common parameter exclusive to the treated and placebo defects is the increase in IL-1ra levels in the early days following defect creation we can assume that IL-1ra was involved in the process. It is possible that the small and short term increase in IL-1ra levels seen in the placebo joints was enough to induce an increase in proteoglycans synthesis or more likely, the combination of the anti-inflammatory properties of IL-1ra and the effects of endogenous IGF-1 was. The fact that the response to IL-1ra and IGF-1 was similar in the placebo and treated joints even if the IL-1ra concentrations were significantly higher for a longer period of time in the latter suggests that high concentrations of those molecules are not necessary to enhance proteoglycans production. It also suggest that IL-1ra mediated effects on proteoglycans synthesis occur in the very early phase of healing and that prolonged protein expression may not be necessary.

We may also speculate that the direct anti-inflammatory effects of IL-1ra are not the only ones responsible for the increase in PGs synthesis. It is possible that the decrease in IL-1 binding to its cell surface receptors associated with the increase in synovial fluid IL-1ra allowed intracellular events leading to an increased anabolic state

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that would have other wise been inhibited by IL-1. Therefore it is possible that IL-1ra rendered the cells more sensitive to IGF-1 and other growth factors synthesized by the cells of the joint or that it led to an increased production of those factors with the end result being an increased in PGs production.

The presence in the repair tissue of the major components of cartilage extracellular matrix was evaluated using immunohistochemistry. Although semi-qualitative, the technique was able to identify difference in protein expression between groups as well as difference in patterns of expression between proteins. It showed an increased presence of type II collagen, aggrecan and S100 in the repair tissue of joints treated with AdEqIL-1ra and AdEqIGF-1 but no change in type I collagen expression. Increase in the signal for aggrecan in the treated group corroborates the results from the biochemical assay. However, the increase in PGs content seen in the placebo group using the DMMB technique is not paralleled by the same increase when aggrecan content is evaluated with IHC. The explanation may reside in the semi-quantitative nature of the technique and the subjective method of evaluation. One study recommends reducing the variability by internally controlling each section by comparing the density of the staining in the repair tissue with that of adjacent normal cartilage and use computer-assisted image analysis to perform the comparison. [355] With the limited amount of tissue available for immuno-staining comparison to normal cartilage was only feasible in a few selected sections in our study but, the use of computer analysis might have help us detect changes in staining intensity not detectable by the naked eye.

Increase in type II collagen production measured by immunochemical techniques or by cyanogen bromide cleavage and separation of marker peptides characterisitic of type I and type II collagen has been described in other studies of cartilage healing; these include microfracture, [181, 182] treatment with growth factors, [242, 354] use of tissue

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grafts, [170] and implantation of cell seeded matrixes. [412, 413] Increasing the amount of type II collagen in repair tissue appears to be easier to achieve than up regulating the production of proteoglycans, which has only been described in a few reports and only when cartilage or implant containing component of the extracellular matrix were used to fill the defect. [412-414] Stimulation of the synthesis of PGs seen in this project was done through the modulation of the metabolism of endogenous cell populations by the short over-expression of factors produced by those same cells. This constitute a major advantage over the other techniques where more complicated and prolong surgeries are necessary to produce a similar effect.

Intensity of type I collagen staining was the same regardless of treatment, indicating that even if IL-1ra and IGF-1 had a beneficial effect on PGs and type II production it was incapable of affecting type I collagen synthesis suggesting that cell differentiation was not complete and a significant number of mesenchymal cells were still present in the defect.

The topographical expression patterns of aggrecan, type I and type II collagen differed from each other but were not affected by treatment. Staining for type I collagen is evenly distributed through out the sections, type II collagen staining is more intense in the deep layer while aggrecan staining is equally seen in both middle and deep layers of the repair tissue. These staining patterns are the same as those published in other studies [181,242, 354, 413] and they seem to indicate that chondrogenesis takes place earlier in the deeper zone of the repair tissue to than move on to the more superficial layers. It also suggests that proteoglycans synthesis may precede type II collagen synthesis. [181] The mechanisms responsible for these healing patterns are still unknown but they probably implicate factors such as distance from the source of mesenchymal cells, sequence of expression and gradients of growth factors in the layers of the repair tissue.

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Gene expression of proteins involved in cartilage healing as well as expression of factors that could be deleterious to the healing process has not been studied in an in vivo situation of cartilage healing. With the help of TaqMan PCR our study look at the expression of some of those genes 16 weeks into the healing of full thickness chondral defects. These results allow a picture of some of the physiologic processes taking place in the joints tissue at that particular time.

After 16 weeks of healing, the only effect of gene transfer therapy on IGF-1 endogenous expression was seen in the repair tissue. Repair tissue from the treated and placebo group expressed significantly less IGF-1 mRNA than the one from the control group. This indicates that even though IL-1ra transgene expression was only elevated for 3 weeks post treatment it seemed to have long term repercussion on IGF-1 expression. The effects are surely indirect and we can only suggest that they might be associated to a more rapid or advance stage of maturation of the repair tissue in those groups. Response to biomechanical stimuli in more mature repair tissue may down regulate IGF-1. This explanation would have more weight if IGF-1 expression in normal cartilage adjacent to the defect would express the same amount of IGF-1 mRNA as the repair tissue but unfortunately this is not the case. An argument could be made that for reasons unknown to us, cells from the repair tissue of placebo and treated joints have diminished IGF-1 synthesis capabilities.

As for IGF-1, IL-1ra expression was decreased in the repair tissue of treated and placebo joints but contrary to IGF-1 it was also diminished in cartilage adjacent to the defect in those same two groups. Again, the extent of this study only allows us to speculate on the possible reasons and mechanisms implicated in these changes of molecular expression of IL-1ra associated to the transgene expression of IL-1ra and IGF-1. The decreased expression was only seen in the horses that received the IA injection of AdEqIL-1ra and AdEqIGF-1 and not in the horse that were never in contact

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with the virus. Therefore it suggests that the decrease in IL-1ra expression may be a negative feedback mechanisms associated with the over expression of viral driven IL-1Ra that took place 8 (treated) to 15 weeks earlier (placebo). On the other hand the mRNA expression does not seem to translate itself into a large amount of protein production since IL-1ra is not detectable in the synovial fluid 4 weeks after infection; therefore, it would be prudent not to over estimate the importance of the difference in IL-1ra mRNA expression reported here.

Unexpectedly, levels of type I collagen expression in repair tissue were extremely low when compared to the levels in cartilage adjacent to the defect used as a control for normal tissue. Since we assumed that samples of cartilage adjacent to the defect were pieces of normal cartilage we did not expect such a high expression in tissue that normally does not express type I collagen. It is possible that some contamination occurred during the processing of the samples or the manipulation of the primers and probe mix, therefore explaining those unusual results. On the other hand it is possible that cartilage adjacent to the defect was undergoing some subtle pathologic process undetected by macroscopic evaluation but visible when analyzed using more sensitive technique like PCR.

The higher level of expression of aggrecan mRNA in synovial tissue when compared to both repair tissue and cartilage adjacent to the defect is hard to explain. Synovium is a connective tissue composed of a thin intima supported by an areolar fibrous stroma composed of type I collagen. The synoviocytes produced hyaluronic acid that is secreted into the synovial fluid but they are not known to be responsible for the production of large quantity of proteoglycans, although no formal studies have been done to confirm that statement. Therefore, the most plausible explanation is that the samples might have been contaminated during the multiple processes involve in getting them ready for PCR evaluation.

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The decrease in type I collagen expression, taken together with levels of type II collagen and aggrecan expression paralleling those of cartilage adjacent to the defect indicates the anabolic state of the repair tissue. It suggests that 16 weeks after wounding, cartilage is still in an active repair phase and is expressing mRNA of proteins typical to hyaline cartilage rather than fibrocartilage or fibrous tissue. These signs of ongoing healing are encouraging and indicate that contrary to what has be postulated before improvement in the quality of repair tissue seems to occur later then 4 months post injury [177, 182,184, 355],. Although signs of anabolic activity are present, catabolic molecules are still being expressed and, the potential for degeneration of the repair tissue or for decrease in anabolic activity is still present. Furthermore, according to our results, those molecules are mainly produced by the repair tissue itself. Effectively, TNFα, MMP3 and MMP13 expression are at their highest in the repair tissue and are relatively low in the synovium and cartilage adjacent to the defects. Surprisingly the expression level of the cytokine IL-1α do not seem to have been up regulated in repair tissue compared to normal cartilage adjacent to the defect.

The information gained by PCR analysis of those tissue samples is very valuable. It indicates that healing of the defect are still in a very active phase and that it is producing the mRNA of the proteins composing hyaline cartilage but that the balance between those anabolic activities and catabolic activities has not been reached since high levels of expression of molecules with catabolic activities is still seen. This means that modulating the events taking place during the first phase of cartilage healing is probably not sufficient to obtain long lasting repair. Inflammatory mediators are still present or can reappear 16 weeks post injury and are endangering the repair process. Efforts to control the expression of those mediators with the use of MMP inhibitors or

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soluble receptors to TNFα may help prevent the damage to the repair tissue seen in long term studies.

The present study allowed us to determined that the gene transfer of AdEqIIL-1ra and AdEqIGF-1 to joints with full thickness chondral defect treated with SBPM was able to increase the amount of PGs and type II collagen present in the repair tissue of the treated defects. However we were unable to determine an effect on clinical parameters of joint disease, macroscopic appearance and histologic composition of the repair tissue. Our inability to detect differences between treated and non-treated joints in those parameters could potentially be explained by factors associated with the study design and the methods of evaluation used to obtain the data. Some of those factors have already been mentioned in the discussion but they are going to be discussed here in more details.

The experimental design called for the use of 16 joints (8 carpi and 8 stifles) in each of the three experimental groups. Previous studies conducted in our laboratory, using the same chondral defect model, showed that significant differences were seen with as little as 12 joints per treatment group, indicating that the number of horses used in the present study should have been sufficient. [181] However, after analyzing the data, important differences in healing were seen between carpal and stifle defects as well as between defects of the same group. These factors increased the standard error of the mean and made it more difficult to obtained significant results especially if the treatment effect was small. Using a larger number of horses could have decreased those errors and make results that were on the brim of being significantly different, different.

Analysis of the lameness data showed that there were no differences in the degree of lameness between the treated and the non-treated joints. It also showed that the average lameness was no more than grade 1 out 5. Very slight signs of lameness

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are hard for an observer to evaluate, especially if the horses are lame on more than one leg. Therefore subjective lameness evaluation might not have been precise enough to detect the subtle gate changes in the horses of this project. A more subjective method of analysis, more particularly, the use of a force plate could have yielded different results.

The number of viral particles injected in the middle carpal joints and the medial femorotibial joints were the same although the volume of the two joints is different. The volume of the middle carpal joint can be estimated at approximately 10 ml and communicates with the carpo-metacarpal joint that normally contains a minimal amount of fluid. The volume of the femorotibial joint can be approximated to be 20 ml but it communicates with the femoropatellar joint that has an estimated volume of 30 ml. [415] These differences in volumes and communication with other joints might have affected the final concentration of transgene proteins in the treated joints. It is plausible that the levels of IL-1ra and IGF-1 present in the middle carpals joint were significantly higher than the ones in the femoro-patellar joints explaining the apparent better healing seen in the carpal defects. Since synovial fluid could not be collected from stifles because of the anatomy of the joint, it will remain unknown if this was the case. However, in face of the improved healing seen in the contralateral non treated joints when compared to the controls, high concentration of IL-1ra may not be necessary to positively affect cartilage healing.

Differences in healing between defects of the same group could have affected results. Differences in healing within a defect could also have had a significant impact on the results. After macroscopic evaluation each defect was divided into predetermined sections, each one assigned to a different assay. Because of the difference in healing within a unique defect, sections of different quality were assigned to each assay performed on that defect. This allowed for the possibility of obtaining different set of

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results for a same defect, potentially explaining why we saw an increase in the PGs content in treated and placebo defects but no change in the proportion of hyaline-like tissue between those and the controls. Since the study was design to collect has much information as possible we had no choice in dividing the repair tissue in multiple sections but, assigning a predetermined section to one particular test would prevent the allocation of better sections to particular tests and diminish the effect of intra-defect variation.

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CHAPTER 5

5 Conclusion

The use of gene transfer to treat or decrease the progression of joint diseases has been the object of research projects for more than a decade but, the interest in its use to enhance cartilage healing is relatively new. Only a small numbers of studies have published *in vivo* work on the subject. [244, 342-347] A smaller number have actually transduced cells with growth factors with the objective to enhance healing of full thickness chondral defects, and those that did, used ex vivo techniques of gene transfer. [244, 346, 247] Our study is the first one to report augmented cartilage healing in chondral defects after the use of an *in vivo* technique to transfer the equine genes of IL-1ra and IGF-1 to horse joints. Although we could not confirm the transgene expression of eqIGF-1 we were able to demonstrate that up regulation of eqIL-1ra expression through gene transfer using an adenoviral vector increased the amount of proteoglycans and type II collagen in repair tissue of full thickness chondral defects treated with subchondral bone plate microfracture. This is the first report of a combined increase in PGs and type II collagen production in a cartilage defect model using a resurfacing technique relying on an endogenous cell population to induce repair. Even if improvement in the biochemical composition of the repair tissue was seen it did not translate into improvement in clinical parameters of pain and disease activity, nor did it improve the macroscopic appearance or composition of the repair tissue.

Proteoglycans expression has been difficult to modulate in *in vivo* model of cartilage healing. Few studies have been able to confirm an increase in proteoglycans

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content following treatments of cartilage defects and the ones that did, used an exogenous source of cartilage or cells to induce healing. [170, 412, 414] Therefore the demonstration that the combination of two relatively simple techniques, microfracture and *in vivo* gene therapy, was able to increase the PGs content in repair tissue represents a significant step forward in cartilage resurfacing research. However a significant increase in PGs and type II collagen content after 16 weeks of healing does not mean reconstitution of normal articular cartilage content and architecture nor is it enough to ensure long term compositional stability and integration of the repair tissue to surrounding cartilage and underlying subchondral bone. Numerous studies have demonstrated evidence of repair tissue degeneration in long term studies using various models and enhancing techniques. [170, 173, 182, 184, 355] Failure of the repair tissue has been associated to the poor capacity of the afore mentioned tissue to sustain the mechanical force induced by normal activities and weight bearing. [184] Overtime, even repair tissue possessing articular cartilage characteristics early one, undergoes degenerative changes characterized by a decrease in PGs and type II collagen content as well as detachment from the subchondral bone. [184] Therefore it is imperative that we pursue our research on techniques like SBPM and *in vivo* gene therapy so we can continue to ameliorate the quality of the repair tissue in chondral defect and created a long lasting, functional surface. The results of this latest study as well as others seem to indicate the need for the use of different anabolic and catabolic factors depending on the stage of healing. The phase immediately after debridement of the defect demands the presence of growth factors with chemotactic abilities and the addition of anti-inflammatory molecules. The next stage would benefit from growth factors with the capacities to induce cell proliferation and differentiation, while later stages seem to require substances influencing cartilage maintenance and substance preventing repair tissue degradation. These are very broad statements and more specific ones will come

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as we continue to decipher and understand the events and identify the endogenous factors involved in cartilage repair.

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6 Tables

Table 1 – Macroscopic evaluation of the repair tissue filling the defects at post-mortem examination (Mean ± SEM) averaged over location and expressed by treatment (grading system: none = 0, slight = 1, mild = 2, moderate = 3, marked = 4)

	colour	**surface irregularities**	**firmness**	**attachment to cartilage**	**attachment to bone**	**volume** (%)
control	mostly white	2.5 ± 0.41	2.75 ± 0.19	2.63 ± 0.34	3.0 ± 0.29	72.25 ± 8.5
treated	mostly white	2.63 ± 0.33	3.19 ± 0.19	2.31 ± 0.31	3.0 ± 0.28	60.31 ± 7.15
placebo	mostly white	2.81± 0.33	3.62 ± 0.19*	1.75 ± 0.31	3.56 ± 0.28	58.75 ± 7.15

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Table 2 – Subjective percentage of the area of repair tissue filling the defects at post-mortem examination (Mean ± SEM) averaged over all treatments and expressed by location.

Site	Area covered (%) ± SEM
Radial carpal bone	84.29 ± 5.53
Femoral condyle	80.75 ± 5.53

Table 3 – Subjective percentage of the area of repair tissue filling the defects at post-mortem examination(Mean ± SEM) averaged over location and expressed by treatment.

Treatment	Area covered (%) ± SEM
Control	87.63 ± 8.43
Placebo	78.57 ± 6.77
AdEqIL-1ra + AdEqIGF-1	81.36 ± 6.77

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Table 4 – Percentages of the different type of tissue filling the defects 16 weeks after surgery and treatment. Expressed as mean ± SEM. Asterisk (*) denotes significant difference within a main effect (joint or treatment), p-value < 0.05.

	Joint		Treatment		
	carpus	stifle	control	treated	placebo
% repair tissue	68.41± 6.38 *	45.85 ± 6.52	64.09 ± 9.27	54.35 ± 7.80	52.94 ± 8.00
% fibrous tissue	29.60 ± 5.71	25.50 ± 5.83	32.05 ± 6.75	26.24 ± 7.03	24.40 ± 7.40
% fibrocartilage	44.95 ± 5.39 *(p=0.07)*	30.48 ± 5.49	36.92 ± 6.36	39.24± 6.62	36.98 ± 6.98
% hyaline-like cartilage	5.85 ± 4.0 *	28.77 ± 4.09	19.72 ± 4.73	14.83 ± 4.93	17.37 ± 5.19
% bone	3.33 ± 2.26	4.87 ± 2.31	0.15 ± 2.99	6.02 ± 2.76	6.12 ± 2.87
% remaining calcified cartilage	3.99 ± 0.81	3.06 ± 0.83	2.82 ± 0.96	4.07 ± 1.00	3.69 ± 1.05
% remodeling bone	15.08 ± 1.98 *	9.77 ± 2.02	10.96 ± 2.73	12.79 ± 2.42	13.79 ± 2.50
% subchondral bone attachment	78.83 ± 6.01 *	54.54 ± 6.15	69.44 ± 7.76	63.68 ± 7.36	66.92 ± 7.67
% cartilage attachment	63.75 ± 9.15	63.33 ± 8.50	63.65 ± 11.04	67.75 ± 11.71	59.23 ± 10.59

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Table 5 - Immunohistochemistry evaluation of the repair tissue collected16 weeks after defect creation (mean ± SEM) averaged over location and treatment and expressed by layers. (Grading system: no staining = 0, slight staining = 1, mild staining = 2, moderate staining = 3, intense staining = 4) Different letters next to the scores represent a statistical difference between treatment group (control, treated and placebo).

	Type I collagen	Type II collagen	Aggrecan
superficial	2.01 ± 0.23	1.63 ± 0.16 [a]	2.09 ±0.14 [a]
middle	2.13 ± 0.23	2.30 ± 0.16 [b]	2.46 ± 0.14 [b]
deep	2.19 ± 0.23	2.88 ± 0.16 [c]	2.71 ± 0.14 [b]

Table 6 - Immunohistochemistry evaluation of the repair tissue collected16 weeks after defect creation (mean ± SEM) averaged over location and layer and expressed by treatments. (Grading system: no staining = 0, slight staining = 1, mild staining = 2, moderate staining = 3, intense staining = 4) Different letters next to the scores represent a statistical difference between treatment group (control, treated and placebo).

	Type I collagen	Type II collagen	Aggrecan
control	1.95 ± 0.28 [a]	2.01 ± 0.14 [a]	2.28 ± 0.18 [a]
treated	2.25 ± 0.24 [a]	2.74 ± 0.15 [b]	2.77 ± 0.15 [b]
placebo	2.13 ± 0.27 [a]	2.04 ± 0.18 [a]	2.11 ± 0.17 [a]

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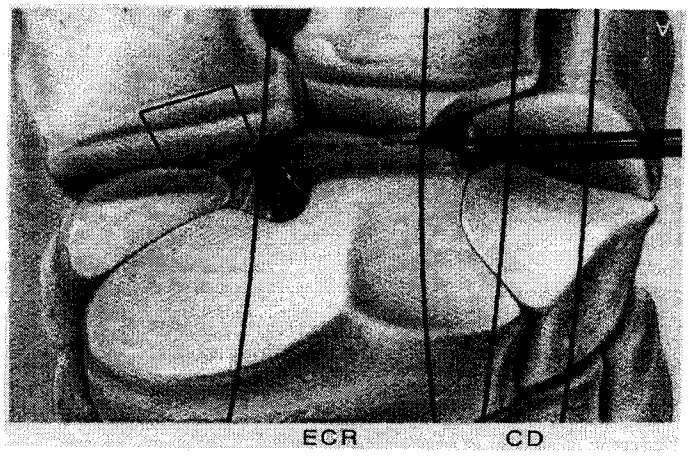
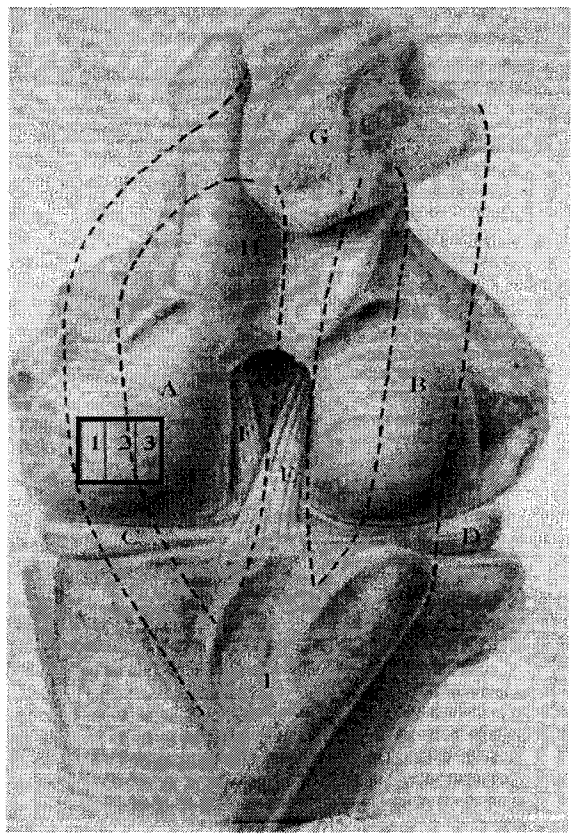
7 Figures



A



B

Figure 1 – Location of the defects in the carpus and stifle. (A) 1 cm^2 full thickness chondral defect created on the lateral weight bearing surface of the distal radial carpal bone. (B) 1 cm^2 full thickness chondral defect in the medial femoral condyle.

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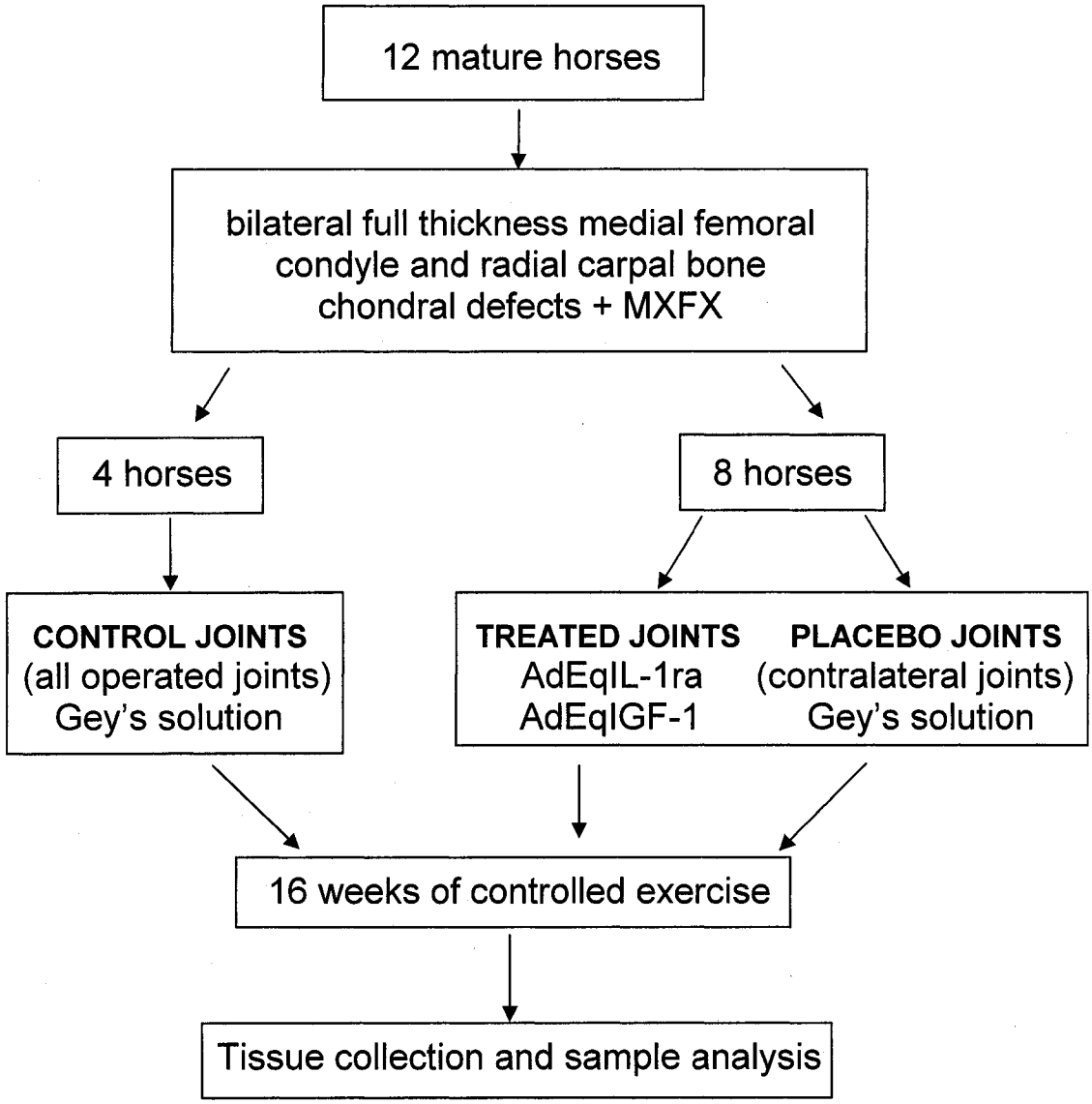

Figure 2 – Experimental design. Full thickness chondral defects were created on the medial femoral condyle and distal radial carpal bone of 12 mature horses. Gey's balanced salt solution was injected in all the operated control joints and in the contralateral joints of the treated joints (placebo joints). The treated joints received the viral preparations AdEqIL-1ra and AdEqIGF-1. Following surgery the horses were placed on a program of controlled exercise for 16 weeks. Horses were euthanized at the end of week 16 and tissues were collected for analysis.

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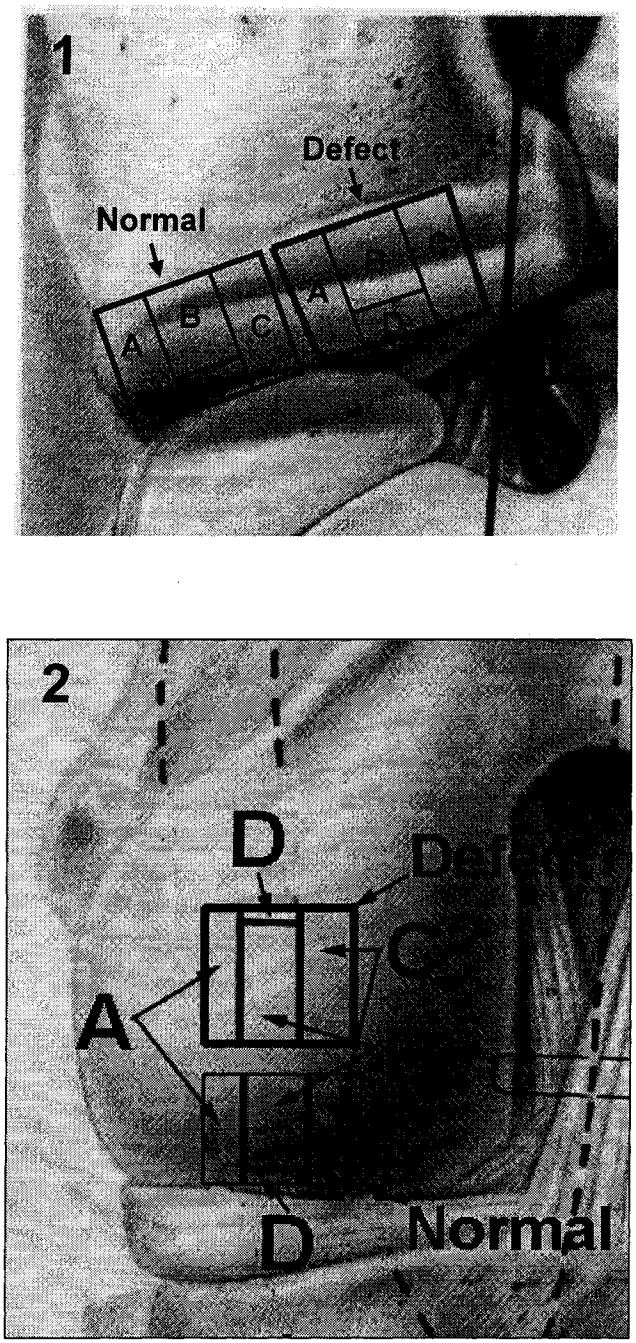

Figure 3 – Specific areas where tissue samples were collected for analysis. (1) Distal articular surface of the radial carpal bone. (2) Articular surface of the medial femoral condyle. A = osteochondral section for histology and histomorphometry. B = cartilage or repair tissue sample for biochemical analysis. C = cartilage or repair tissue sample for immunohistochemistry. D = cartilage or repair tissue sample for PCR analysis.

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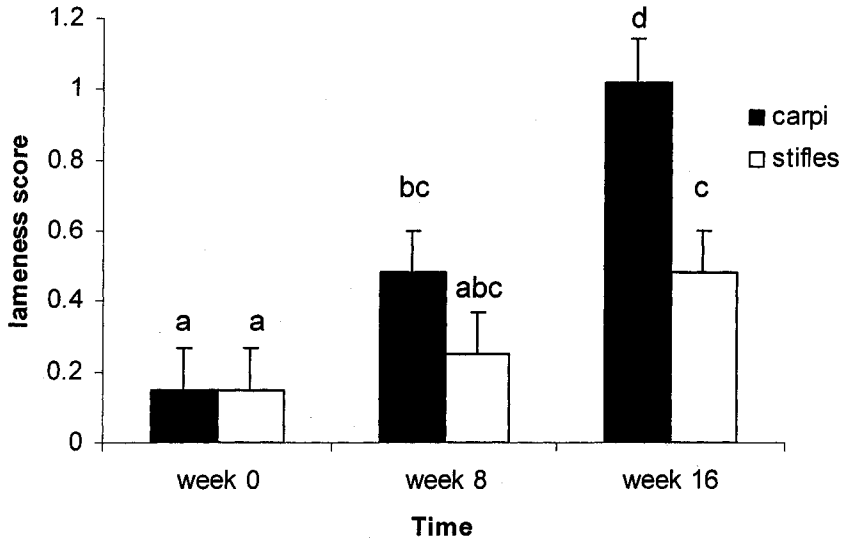

Figure 4 - Lameness scores averaged over treatment groups plotted over time. Different letters indicate a statistical difference between bars. (P – value < 0.05)

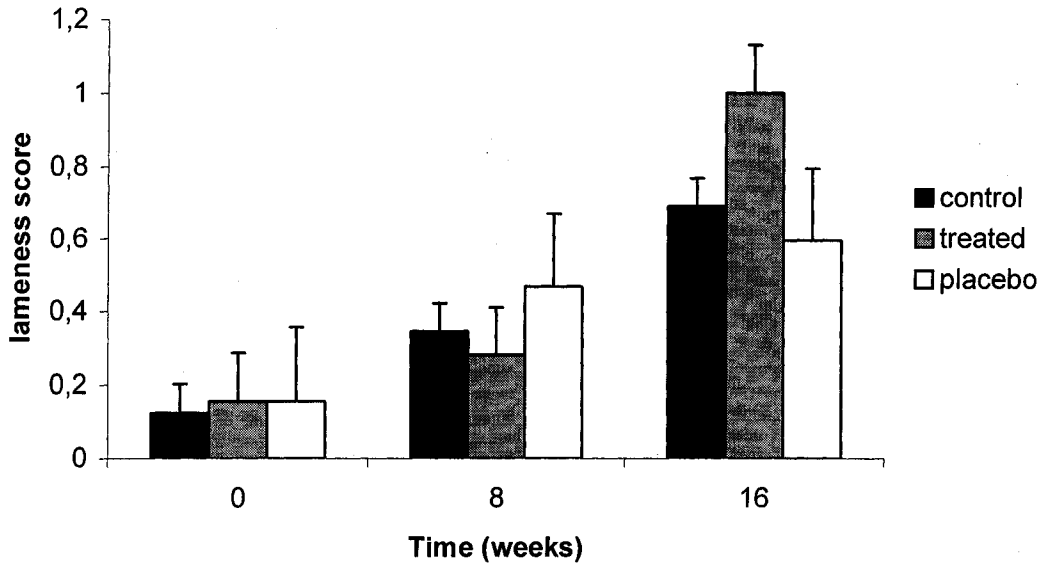

Figure 5 – Lameness scores averaged over location and plotted over time. At the beginning of project the degree of lameness was similar for all horses. Eight weeks after defect creation, the lameness scores had increased but did not significantly differ between groups. There was still no difference between the degrees of lameness in each group at week 16, although the severity of lameness had increased from the previous evaluations.

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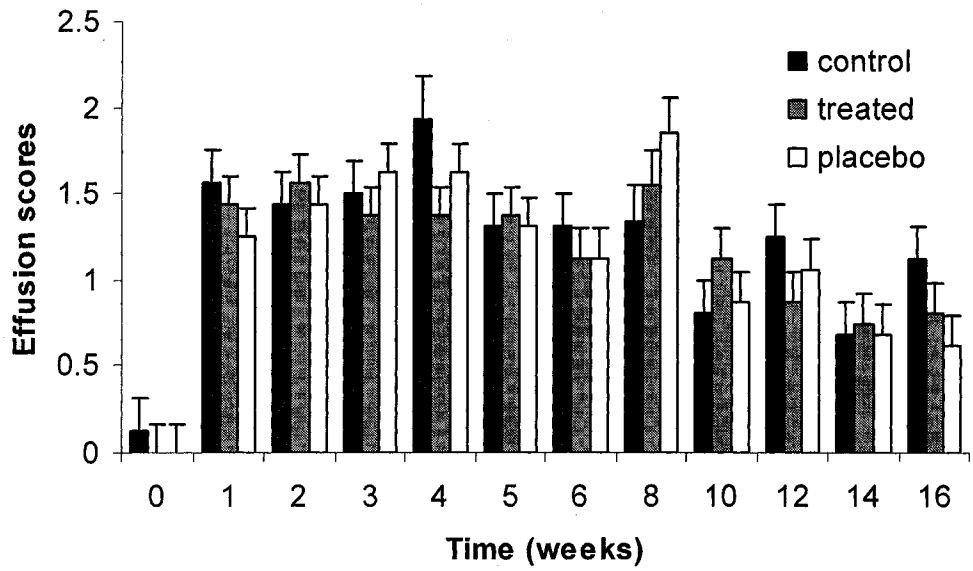

Figure 6 – Effusion scores of carpi and stifle joints plotted over time averaged over locations.
Effusion scores were all significantly increased after surgery and remained above baseline levels until the end of the study on week 16. A significant decrease from the previous weeks was seen on week 10 and persisted until week 16.

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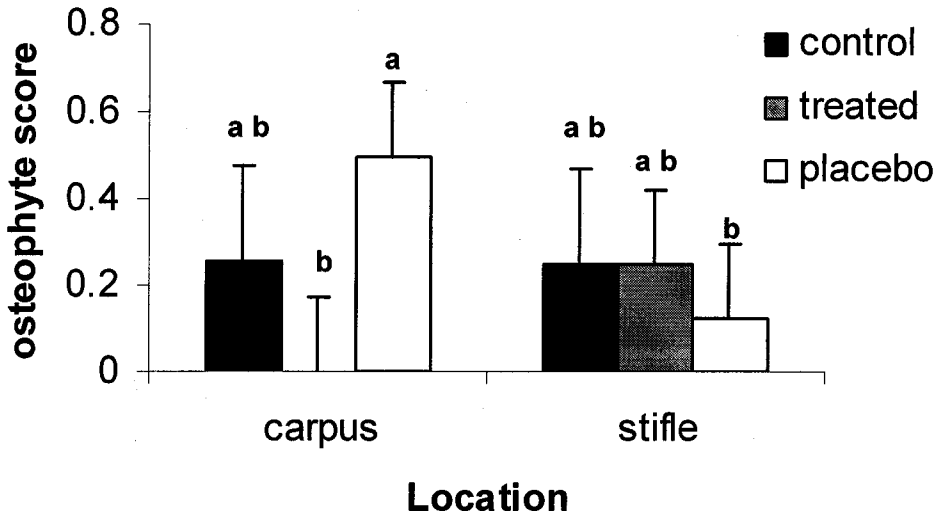

Figure 7 – Osteophyte formation scores 16 weeks post-surgery in the three treatment groups plotted by location. Different letters represent statistical difference (p-value < 0.05) between bars.

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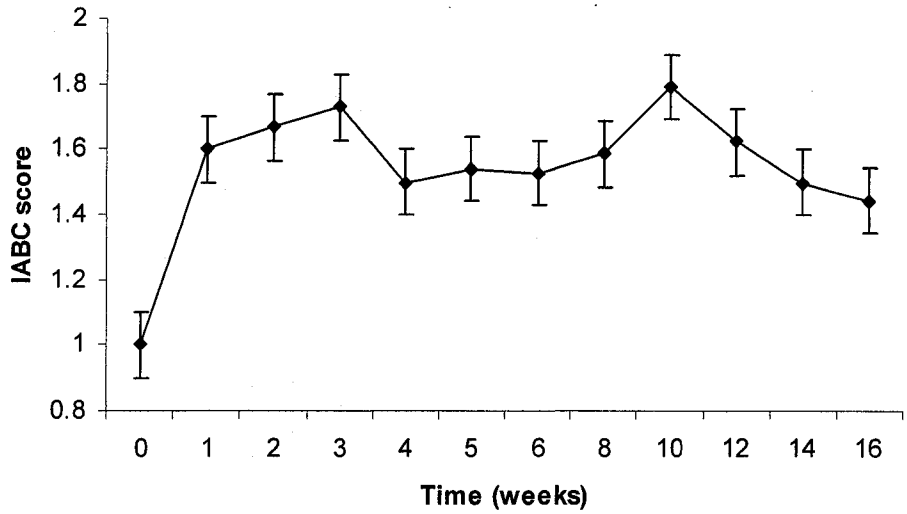

Figure 8 – Iatrogenic blood contamination (IABC) scores of synovial fluid collected from the carpi averaged over treatments and plotted over time.
As significant increase in IABC was seen between week 0, when collection was performed under general anesthesia and the following weeks when it was performed on standing horses.

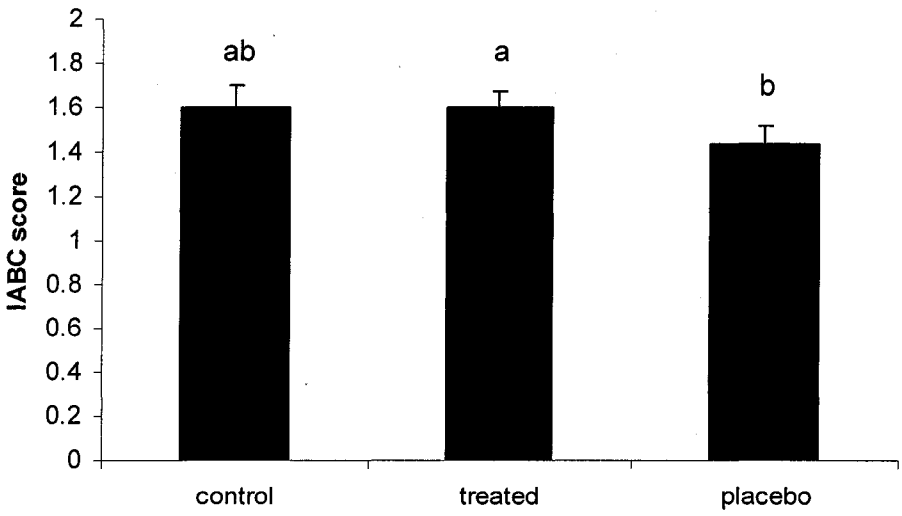

Figure 9 – IABC scores of synovial fluid collected from the carpi averaged overtime plotted by treatment. Different letters represent a statistical difference between groups (P – value
< 0.05).

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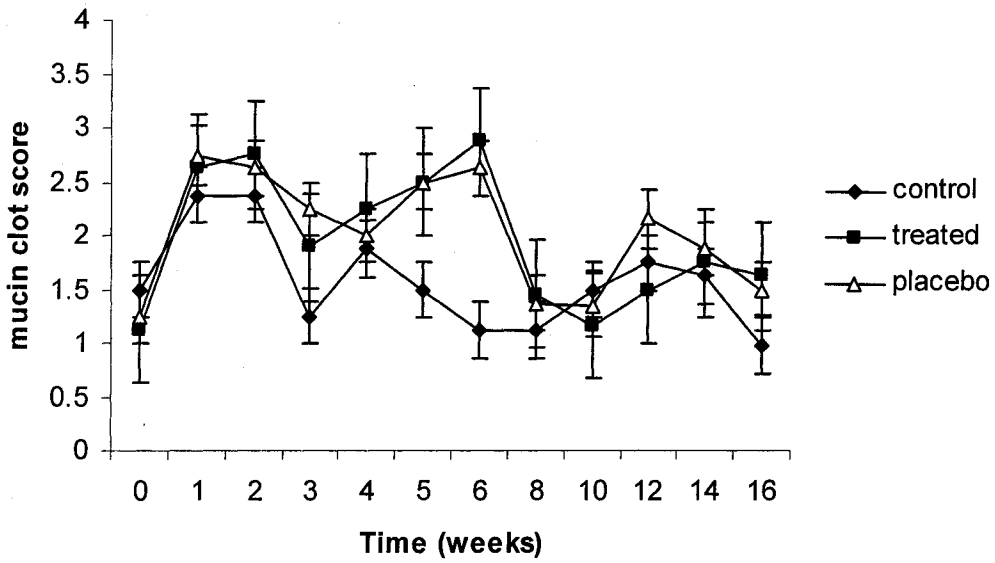

Figure 10 - Mucin clot scores of middle carpal joint synovial fluid plotted overtime. Defect creation and treatment significantly increased the mucin clot score. Mucin clot quality in the control joints returned to normal by week 3. Mucin clot quality in the treated and the placebo groups remained elevated above pre-surgical levels until week 8.

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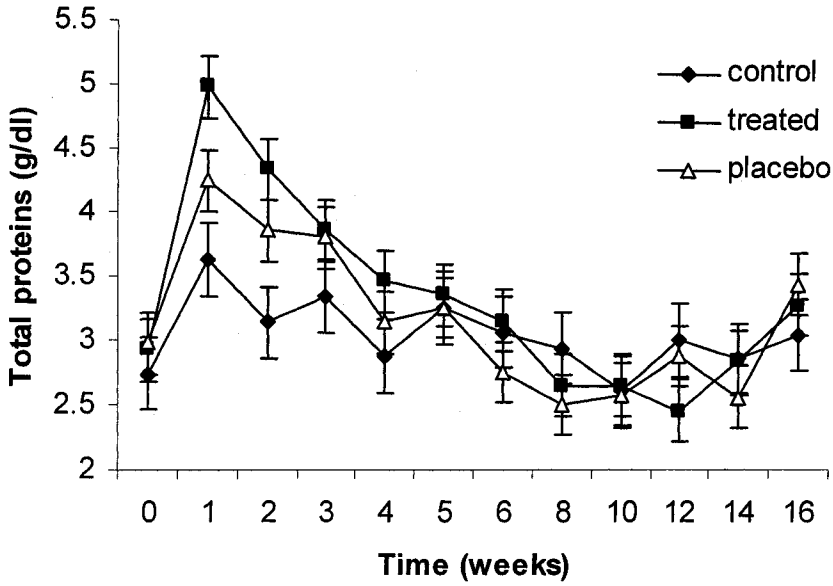

Figure 11 – Synovial fluid total proteins plotted over time.
Surgery and treatment significantly increased total proteins concentration in all groups. At week 1 post surgery the increase in treated the joints was significantly greater than in the placebo and control joints. Week 2 post surgery, the total proteins concentration in the treated joints was still significantly higher than in the control joints. Total proteins concentration return to baseline level on week 2 for the control joints, on week 4 for the placebo joints and on week 5 for the treated joints.

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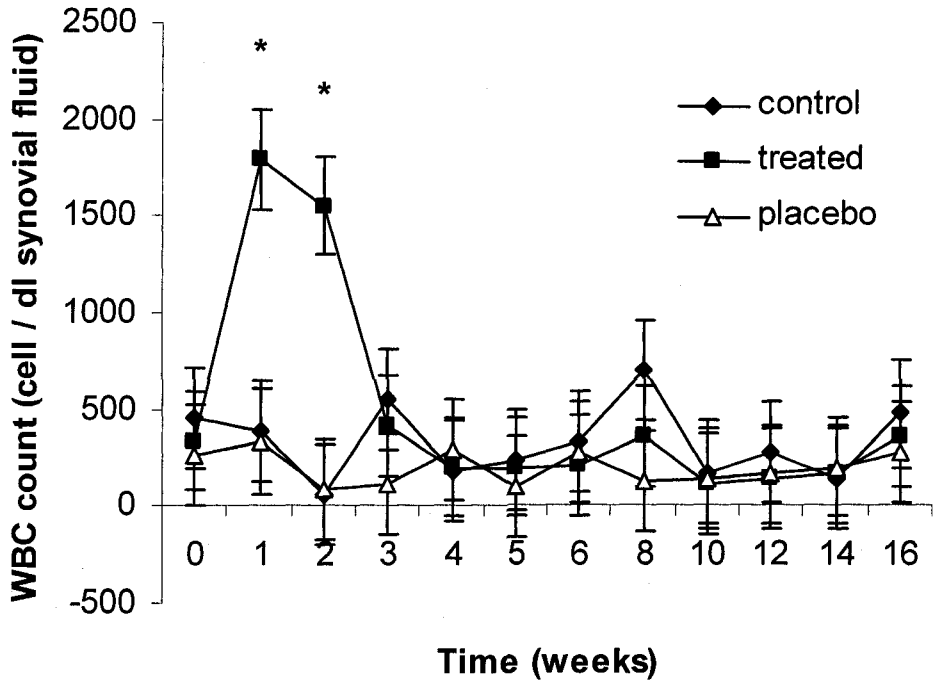

Figure 12 - WBC counts measured in synovial fluid of radial carpal joints plotted over time. An asterisk (*) denotes a statistical difference (p-value < 0.005) between the selected data points and all other data points in the same time period.

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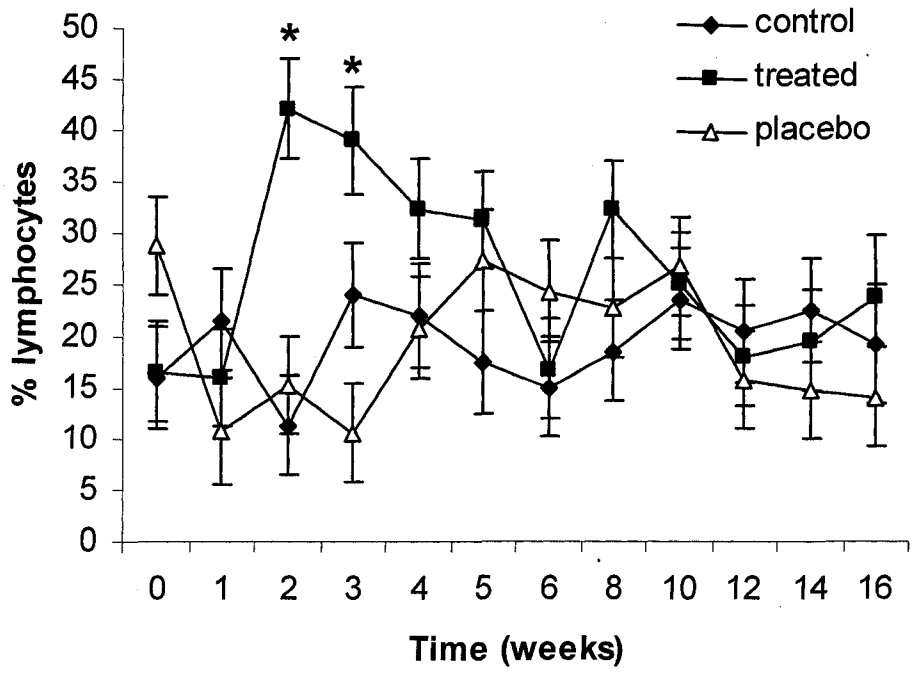

Figure 13 - The percentage of lymphocytes in the synovial fluid of radial carpal joints plotted over time. An asterisk (*) denotes a statistical difference (p-value < 0.05) between the selected data points and all other data points in the same time period.

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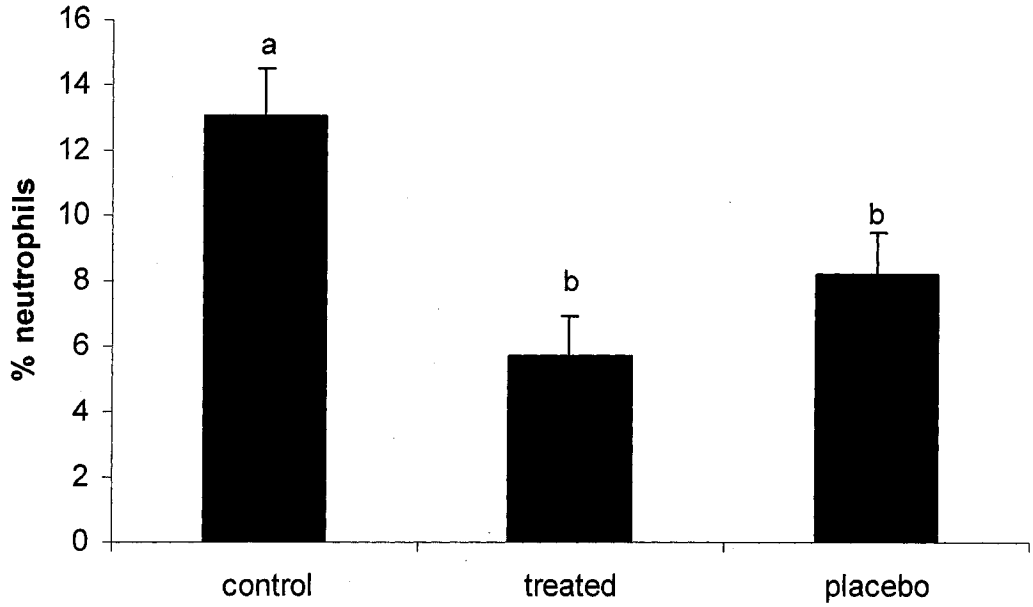

Figure 14 - The percentage of neutrophils in the synovial fluid of radial carpal joints plotted by treatment averaged over time. Different letters represent a statistical difference between groups (P – value < 0.05).

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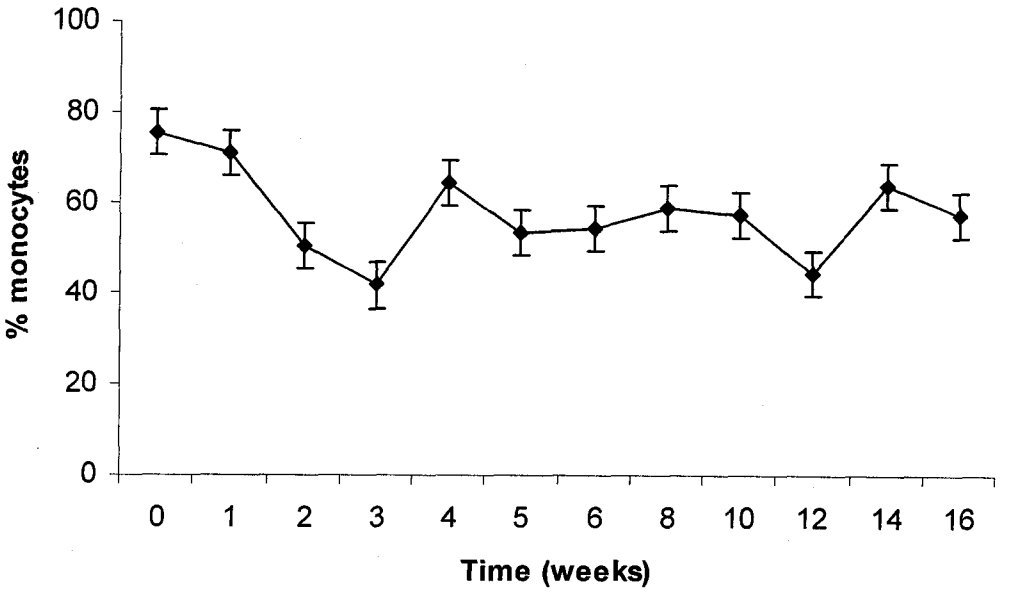

Figure 15 – Percent of monocyte in the synovial fluid of radial carpal joint plotted over time, averaged over treatments.

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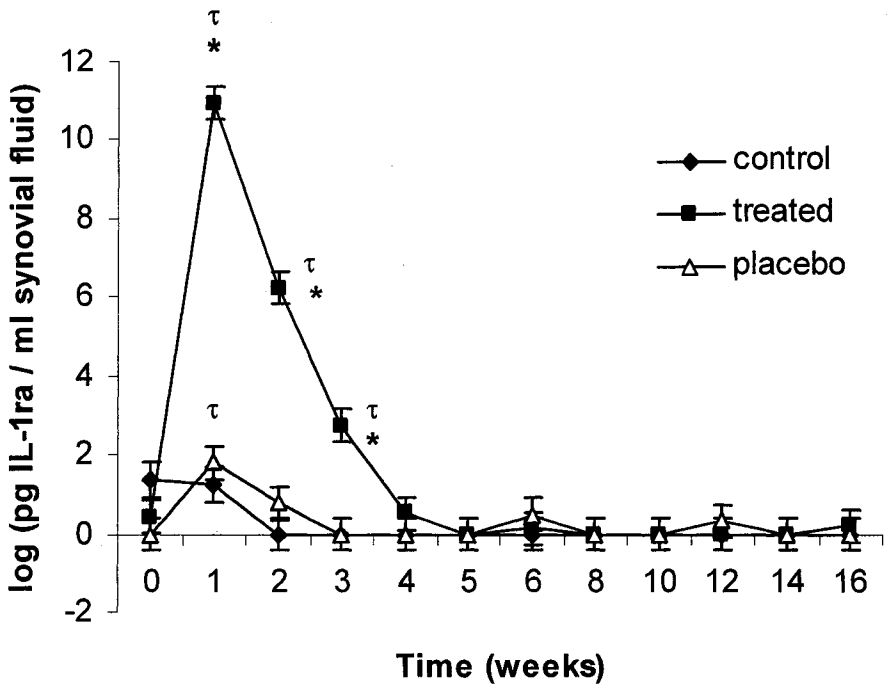

Figure 16 – Log of IL-1ra concentration in synovial fluid collected from radio-carpal joints plotted over time. An asterisk (*) denotes a statistical difference (p-value < 0.05) between the selected data points and all other data points in the same time period. The Greek letter tau (τ) denotes a statistical difference (p-value < 0.05) between the selected data points of a group and the base line value of that particular group.

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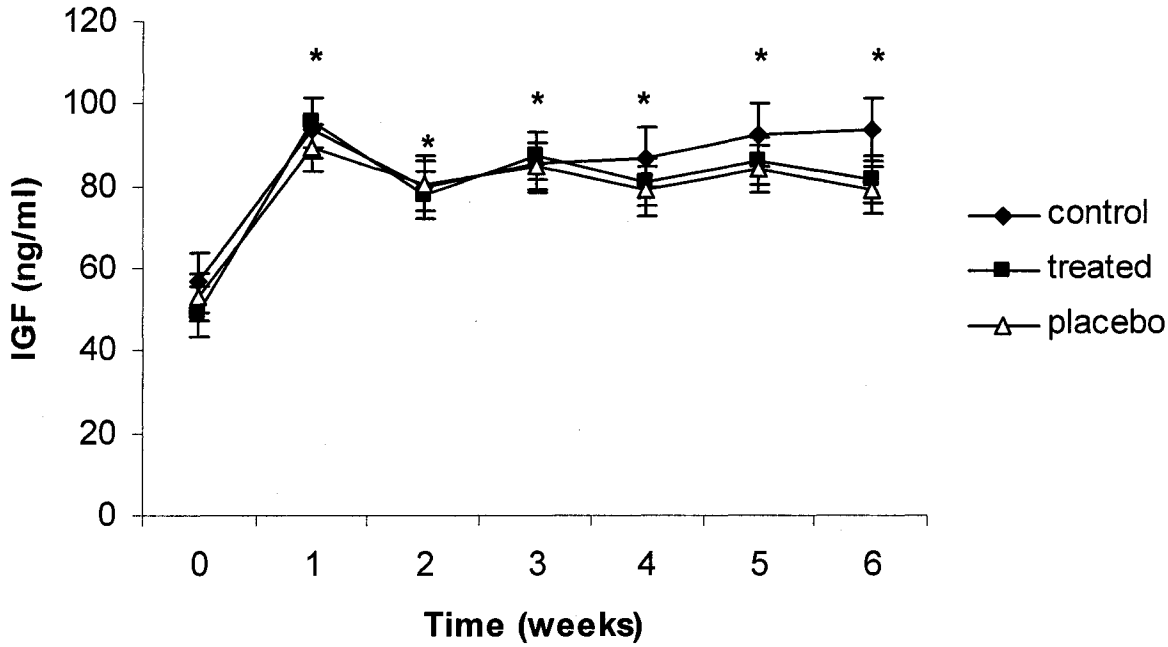

Figure 17 - IGF-1 concentration in carpal synovial fluid plotted over time. An asterisk (*) denotes a significantly different from baseline concentrations at week 0 (p-value < 0.05).

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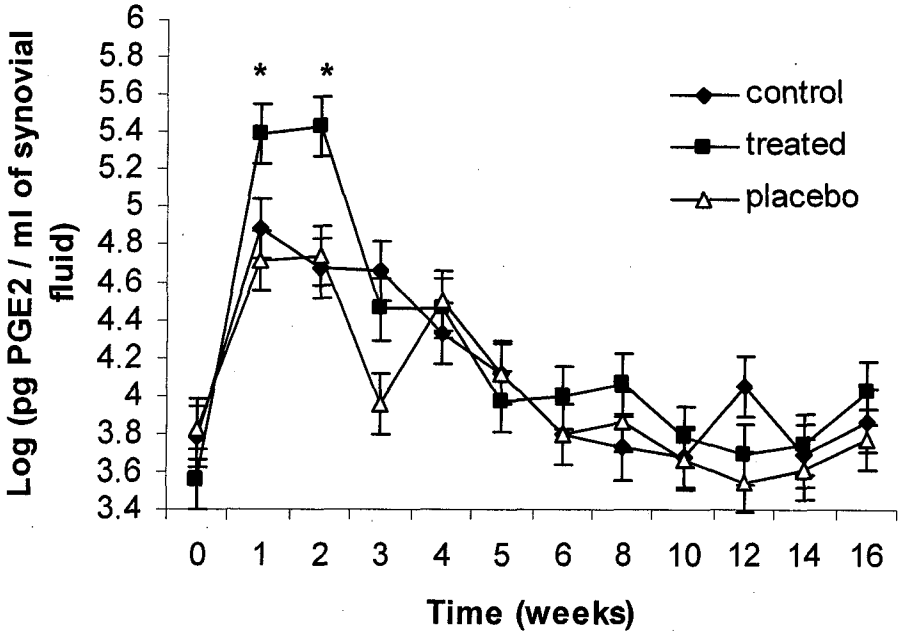

Figure 18 - Log of PGE2 concentration measured in synovial fluid of middle carpal joints plotted over time. An asterisk (*) denotes a statistical difference (p-value < 0.05) between the selected data points and all other data points in the same time period.

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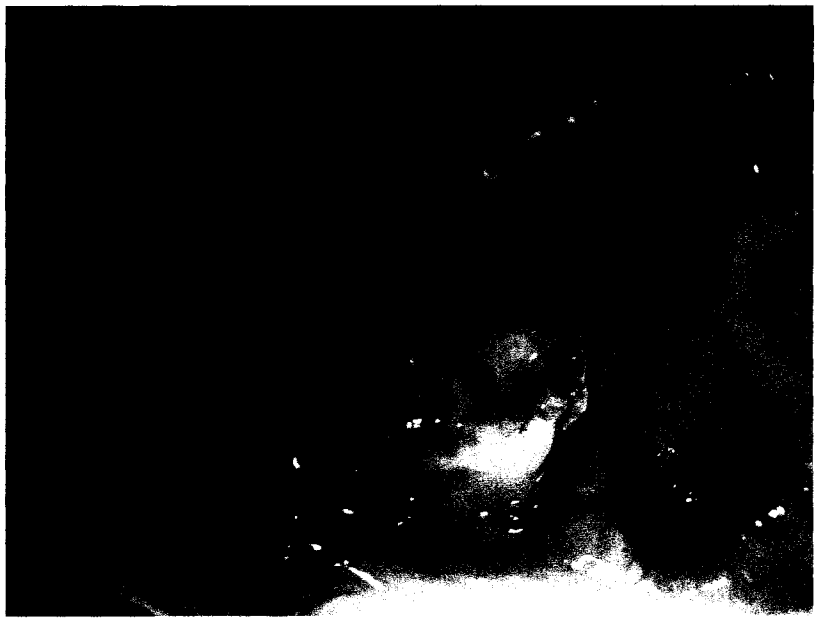

Figure 19 - Photograph of a right radial carpal bone defect from a control joint with focal red area in the repair tissue associated to the presence of vascularization

Figure 20 - Photograph of a right femoral condyle defect from a placebo joint showing red area of repair tissue associated with hemorrhage.

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Figure 21 – Photograph of a right radial carpal bone defect from a control joint filled with relatively uniform repair tissue.

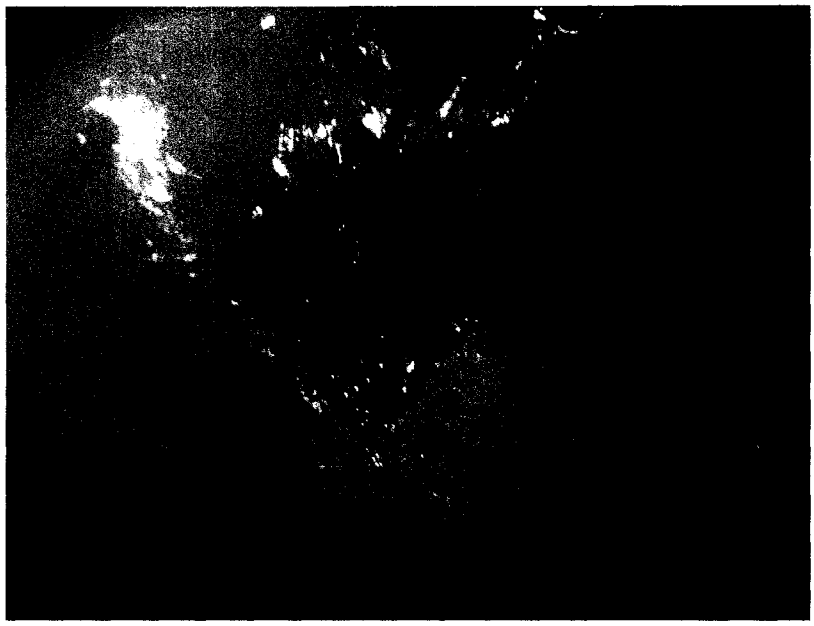

Figure 22 – Photograph of a left femoral condyle defect from a placebo joint showing repair tissue with pits that seem to correspond to microfracture holes.

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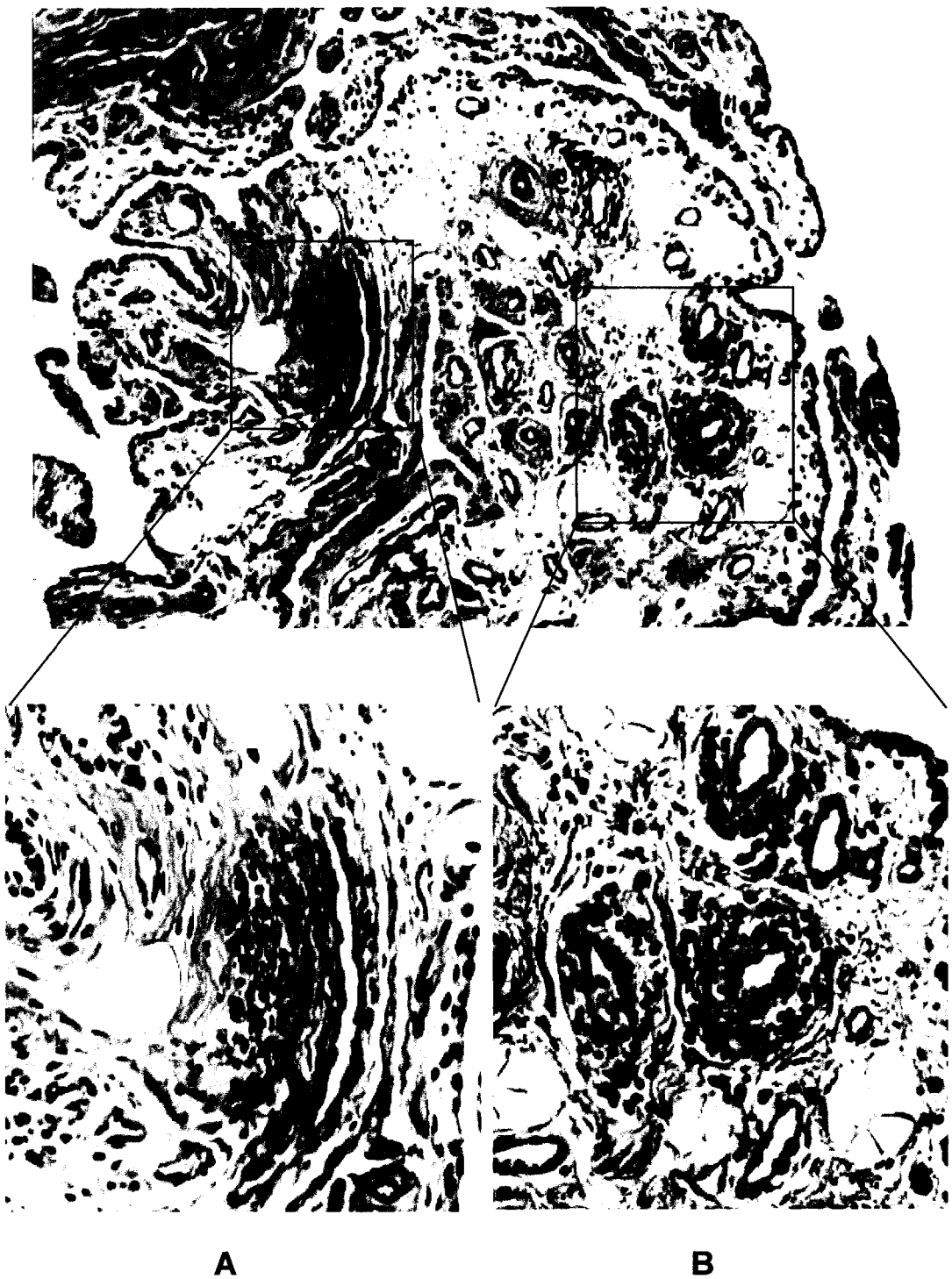

Figure – 23 Cellular infiltration of the synovial membrane from a carpus treated with AdEqIL-1ra and AdEqIGF-1. (A) Foci of cellular infiltration in the subintima. (B) Perivascular cellular infiltration. Magnification: 100X and 200X. Section stained with H & E.

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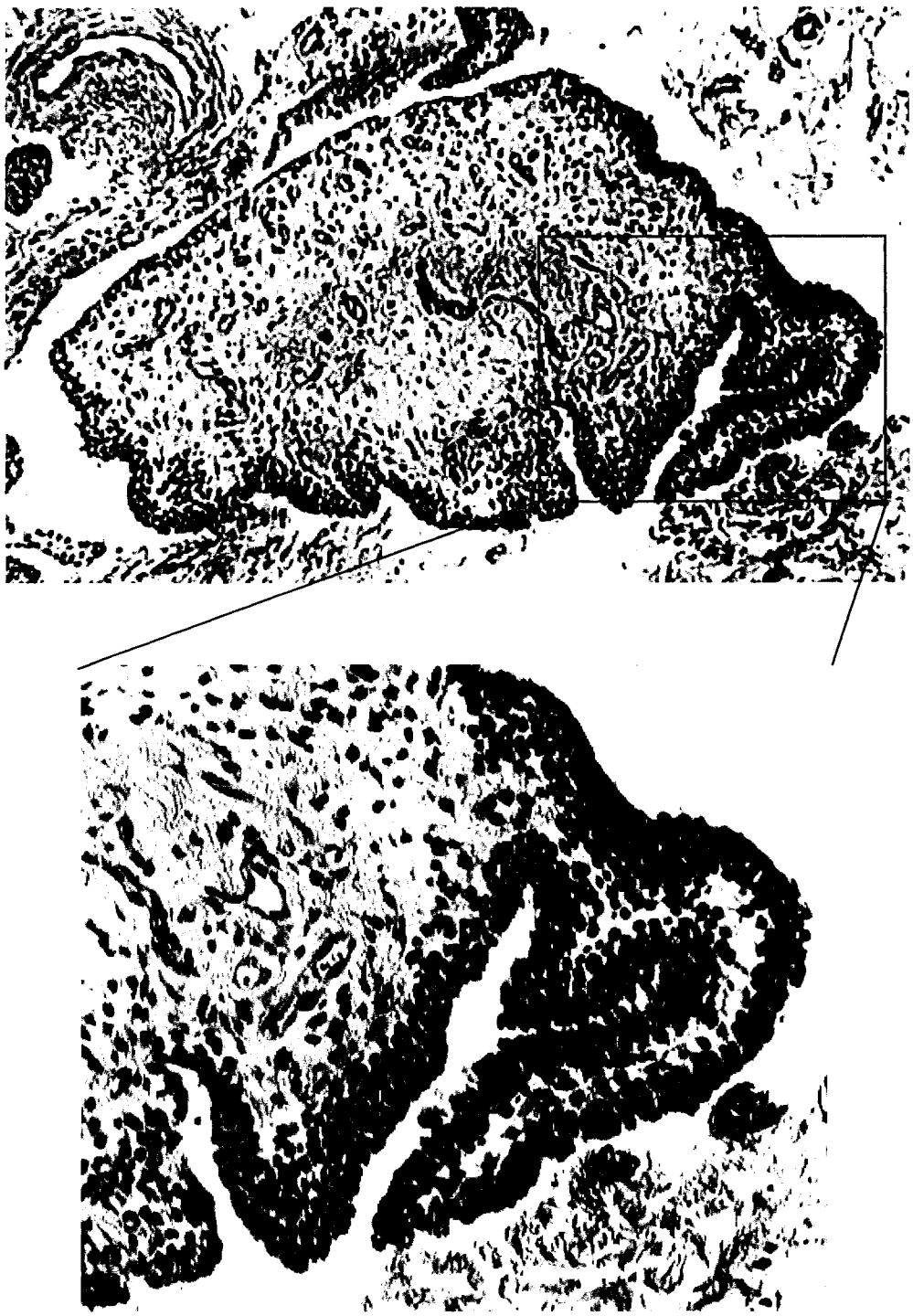

Figure – 24 Intimal hyperplasia of the synovial membrane of a carpal joint treated with the placebo. Magnification: 100X and 200X. Section stained with H & E.

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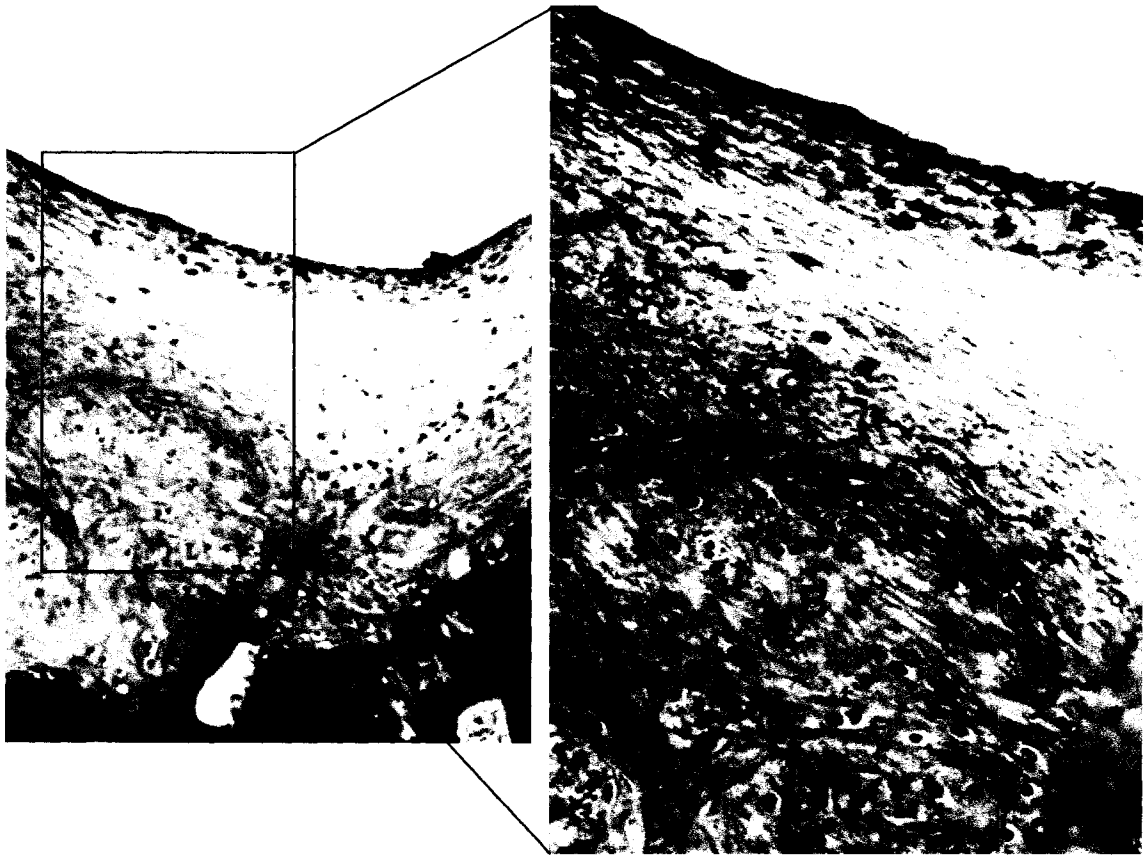

Figure – 25 Osteochondral section from a femoral condyle of a joint treated with AqEqIL-1ra and AdEqIGF-1 stained with H & E showing a portion of the repair tissue. (A) Fibrous tissue characterized by cells with a fibroblastic appearance with a fusiform shape and collagen fibers orientated parallel to the surface. (B) Fibrocartilage characterized by the presence of dense collagen fibers with a random orientation than in and by rounded cells surrounded by a thin layer of pericellular matrix. Magnification 100X and 200X.

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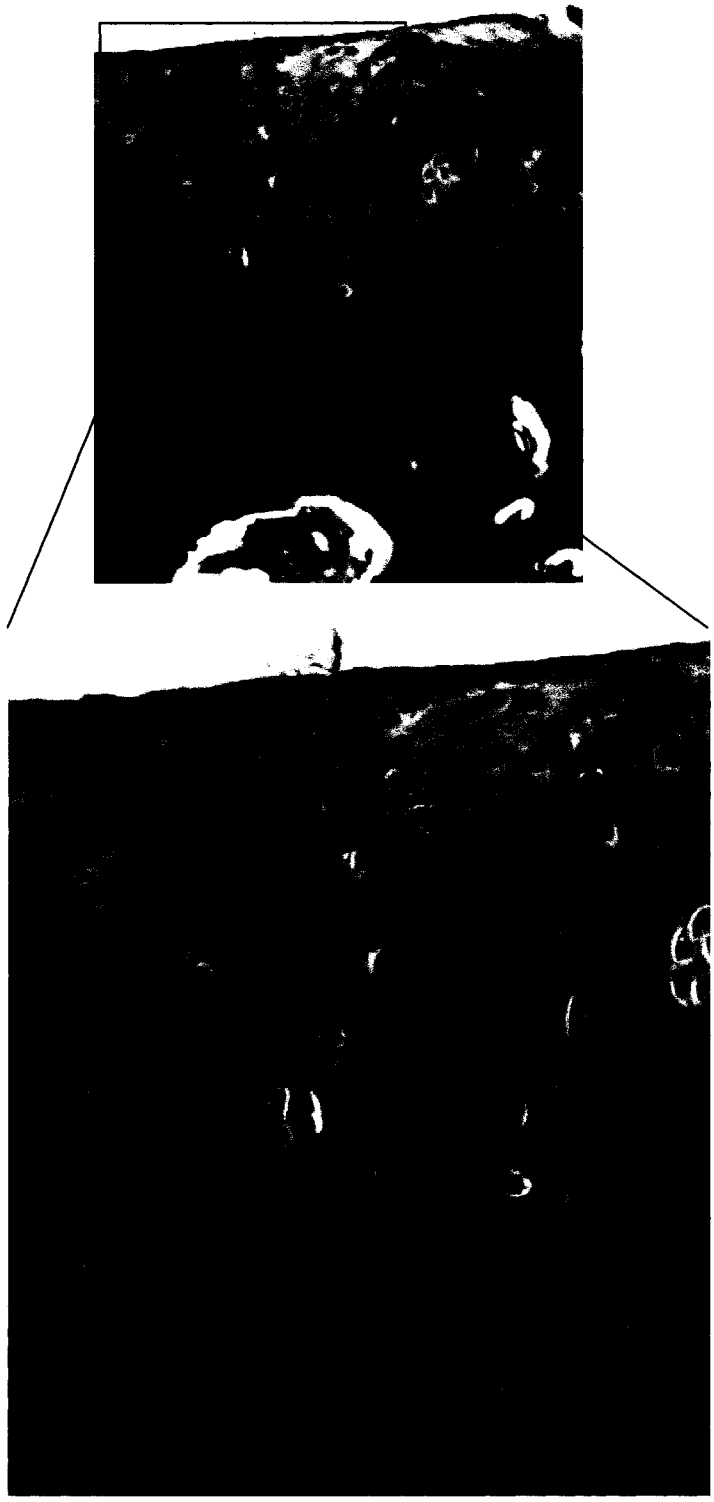

Figure – 26 Osteochondral section from a radial carpal bone from a joint in the control group stained with H & E showing a portion of the repair tissue with hyaline- like cartilage. Islands of hyaline-like cartilage characterized by multiple small clusters of chondrocytes surrounded by pericellular and some interterritorial matrix. Magnification 100X and 200X.

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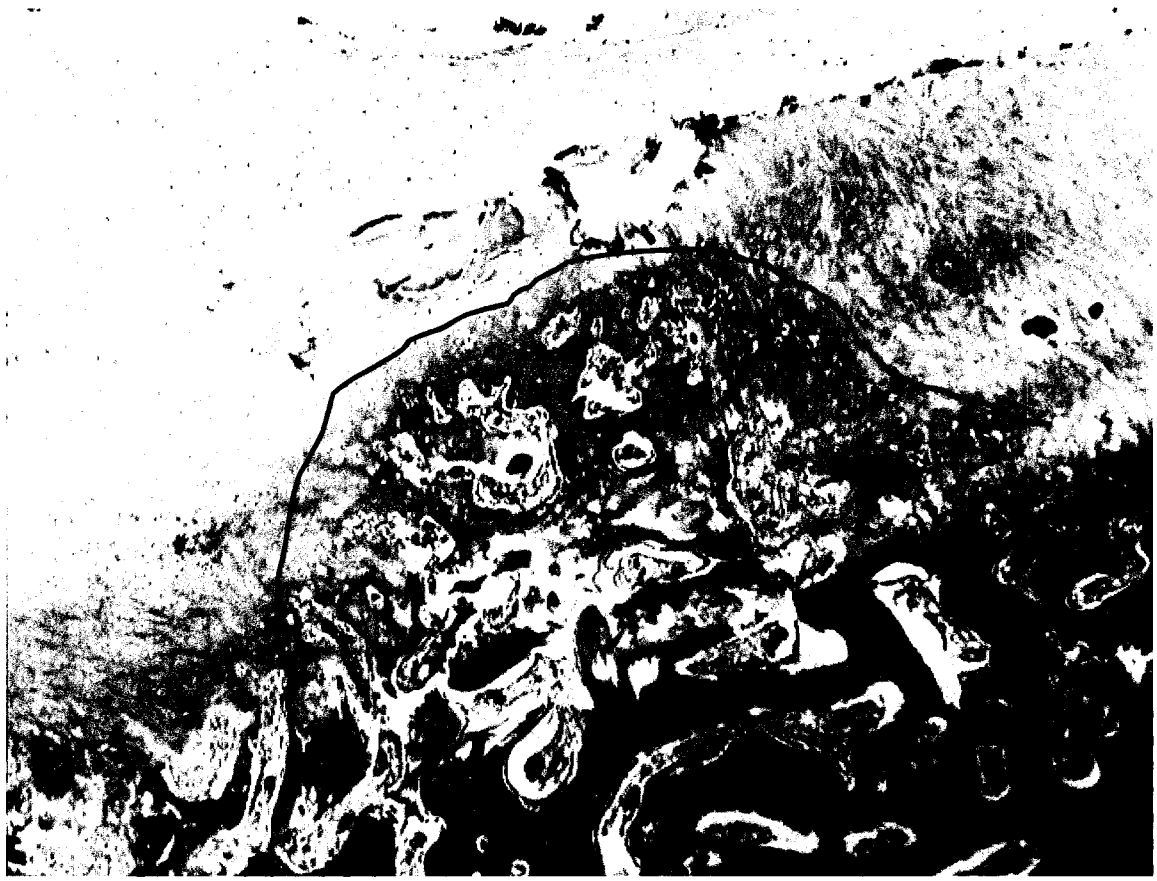

Figure – 27 Osteochondral section of a femoral condyle of a joint treated with AqEqIL-1ra and AdEqIGF-1 stained with H & E showing a portion of the repair tissue present in the defect. (A) The delineated region shows bone invading the bottom of the defect. (B) Subchondral bone (C) Fibrocartilage. Magnification 40X.

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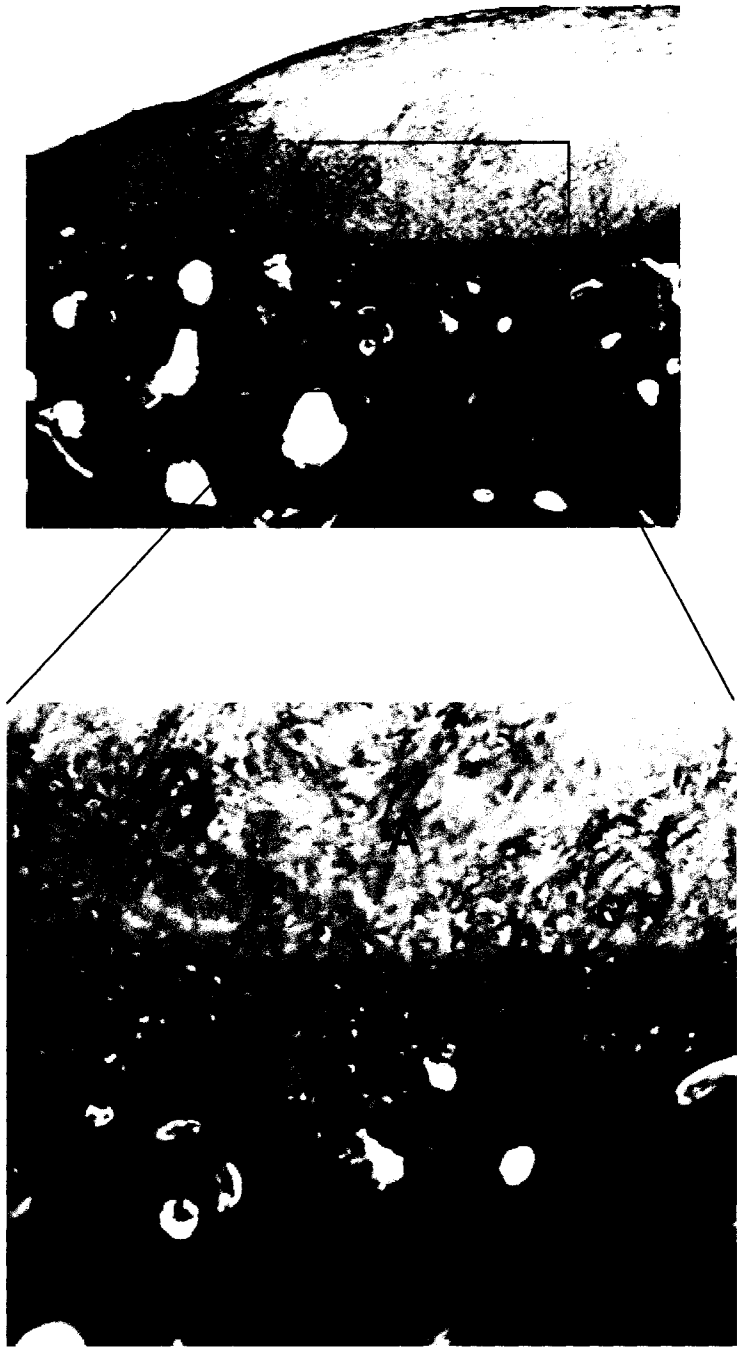

Figure – 28 Osteochondral section of a radial carpal bone from a joint in the placebo group stained with H & E showing a portion of the repair tissue present in the defect. (A) Area of remodeling characterized by mineralization of cartilage and new bone formation at the interface of repair tissue (B) and subchondral bone (C) Magnification 100X and 200X.

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A B

Figure 29 - Immunostaining for type I collagen protein in 16-week repair tissue from a treated femoral condyle. (A) Type I collagen (brown color) is distributed evenly throughout the extracellular matrix of the repair tissue section. (B) Negative control; the type I collagen antibody was pre-absorbed with horse serum before being applied to the section. Magnification 40X.

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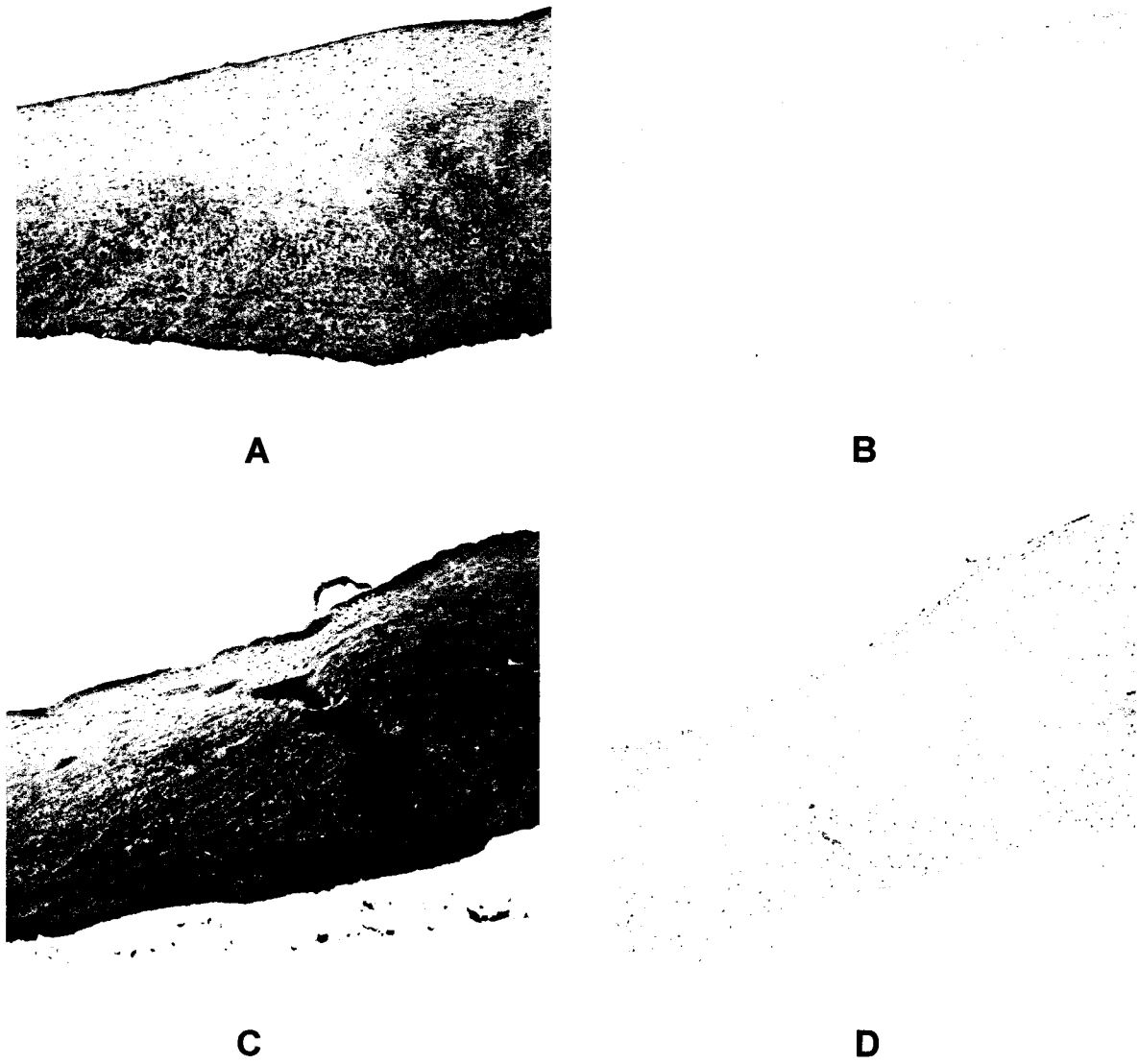

Figure 30 – Immunostaining for type II collagen protein in 16-week repair tissue. A) Repair tissue from the control group B) negative control of A). C) Repair tissue from the treated group D) Negative control of C). In A and C, staining for type II collagen (brown color) is more intense in the deep layer of the repair tissue and almost completely absent in the superficial layer, also staining intensity is greater in the treated group than in the control one. For the negative controls, the type II collagen antibody was pre-absorbed with antigen before being applied to the section. Original magnification 100X.

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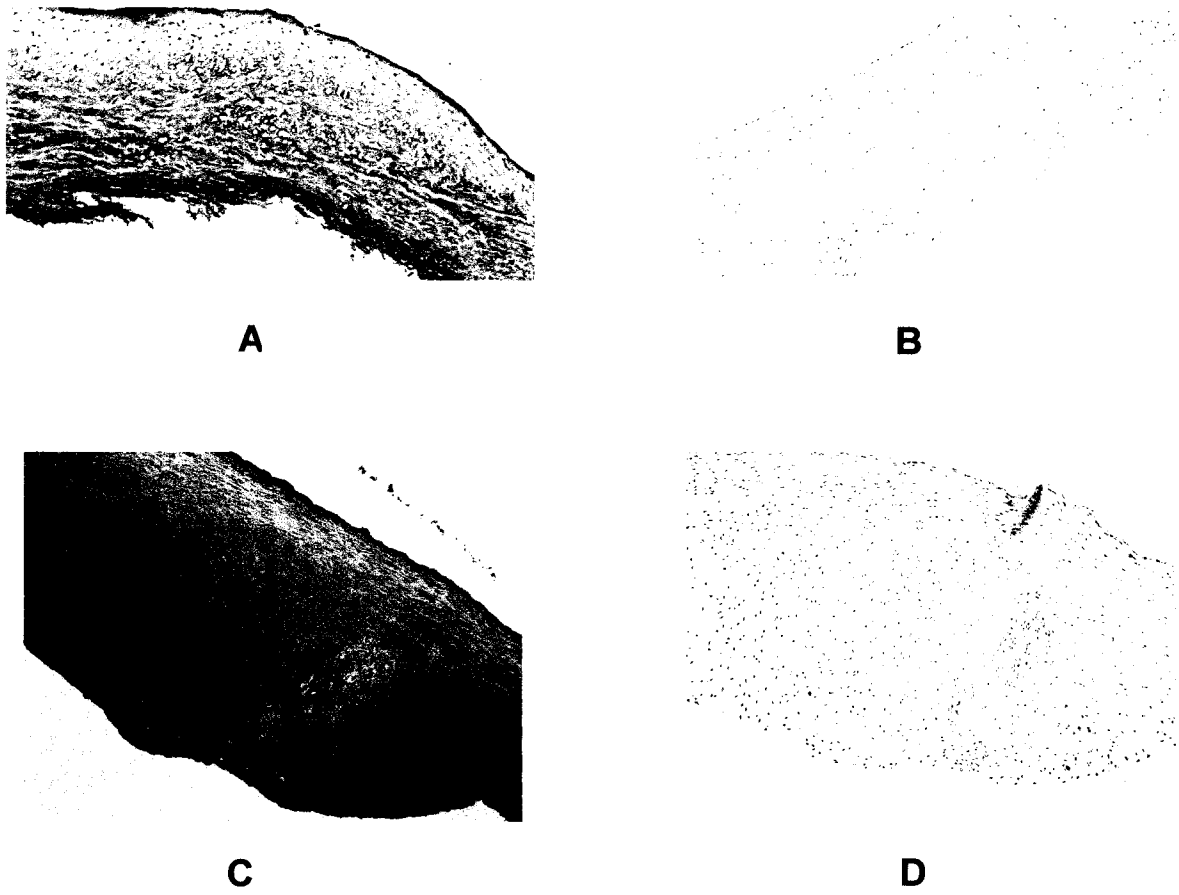

Figure 31 – Immunostaining for aggrecan protein in 16-week repair tissue. A) Repair tissue from the control group B) negative control of A). C) Repair tissue from the treated group D) Negative control of C). In A and C, staining for aggrecan (brown color) is more intense in the deep and middle layers of the repair tissue and almost completely absent in the superficial layer, also staining intensity is greater in the treated group than in the control one. For the negative controls, the aggrecan antibody was pre-absorbed with horse serum before being applied to the section. Original magnification 100X.

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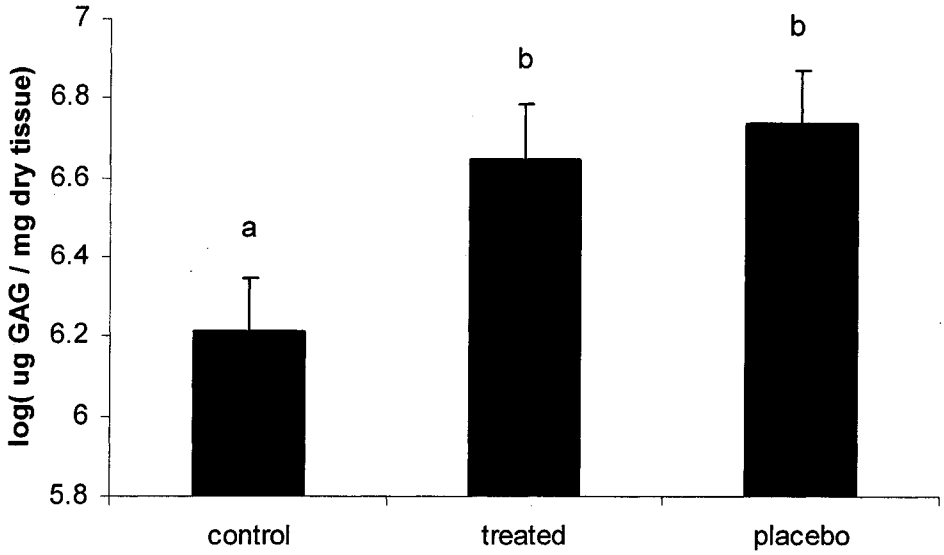

Figure 32 - Log of GAG content in repair tissue present in defects of control, treated and placebo joints. Different letters represent a statistical difference between groups. (p-value < 0.05).

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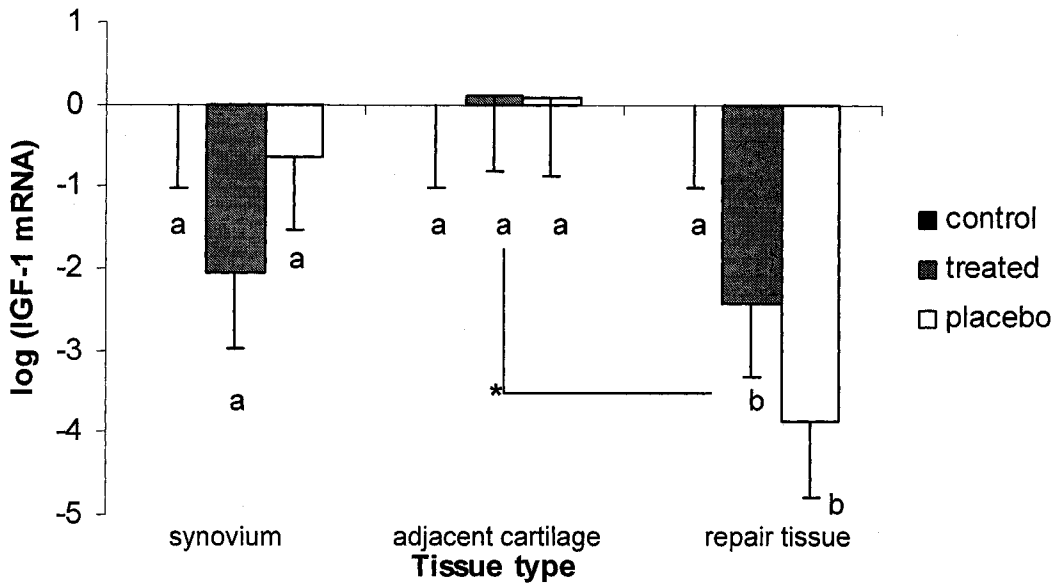

Figure 33 - Log of IGF-1 mRNA expression plotted by tissue type. Different letters represent a statistical difference within a same tissue type and between groups (P – value < 0.05). The lines and asterisk(*) between linking the two selected data points denote a statistical difference (p-value < 0.05) between the two points.

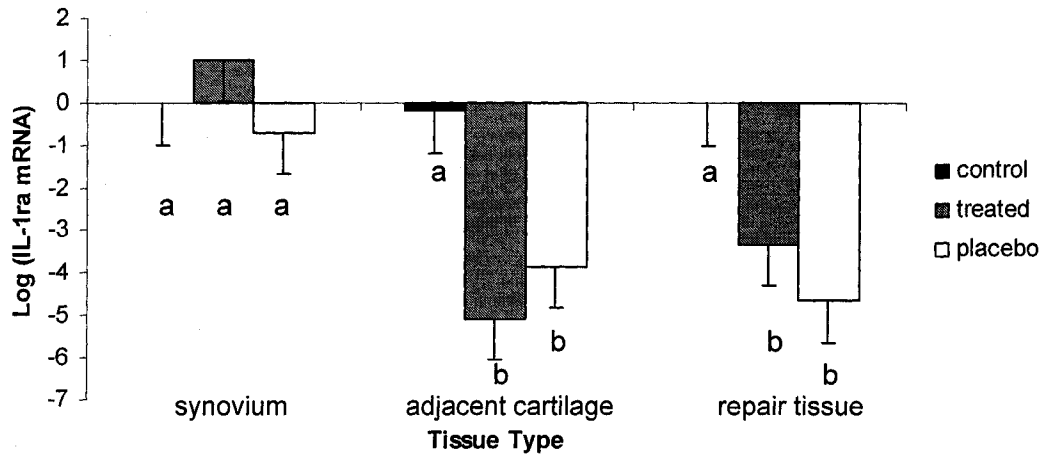

Figure 34 - Log of IL-1ra mRNA expression plotted by tissue type. Different letters represent a statistical difference within a same tissue type and between groups (p-value < 0.05).

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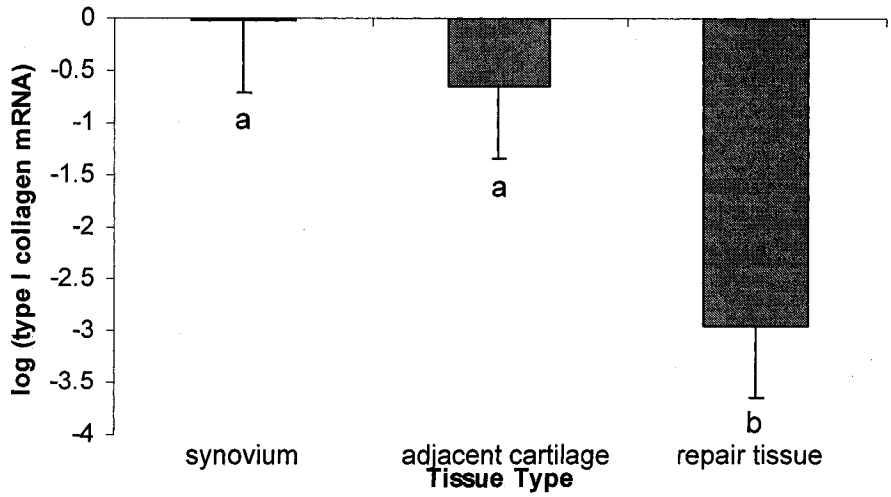

Figure 35 - Log of type I collagen mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

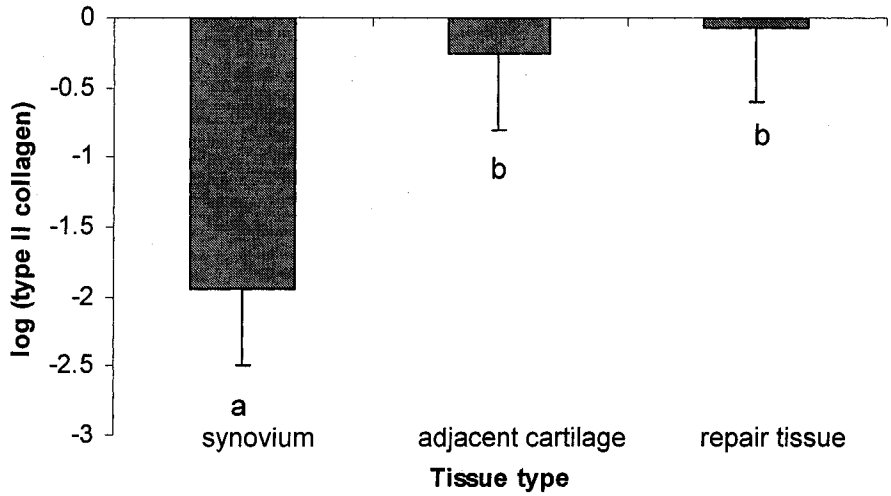

Figure 36 - Log of type II collagen mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

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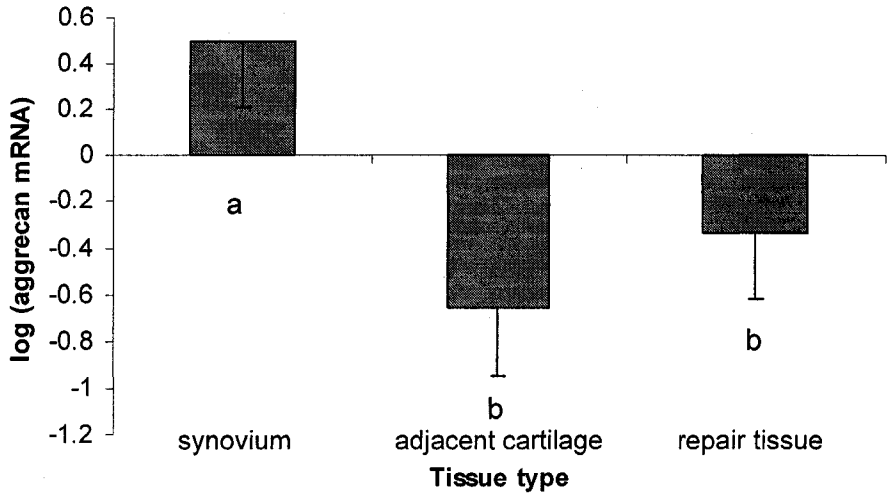

Figure 37 - Log of aggrecan mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

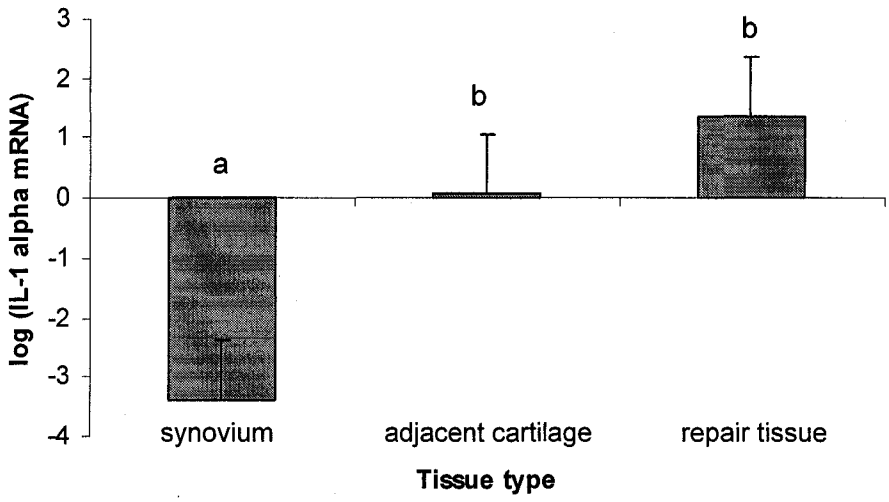

Figure 38 - Log of IL-1α mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

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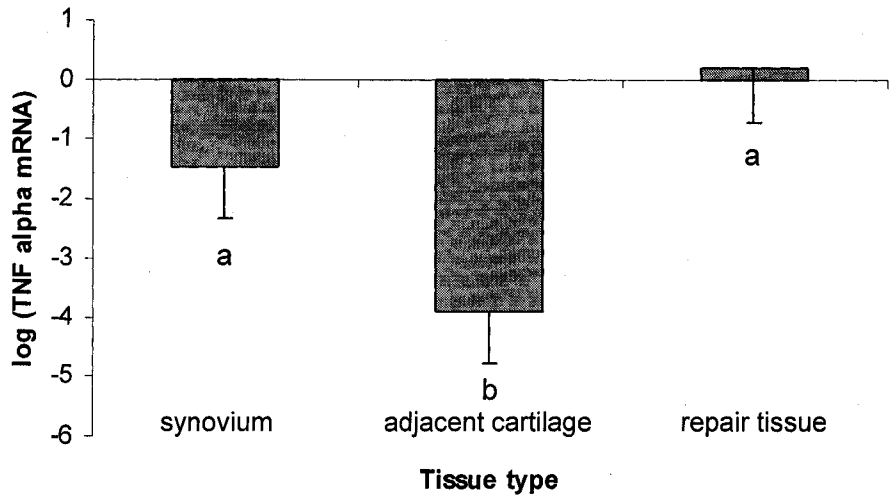

Figure 39 - Log of TNFα mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

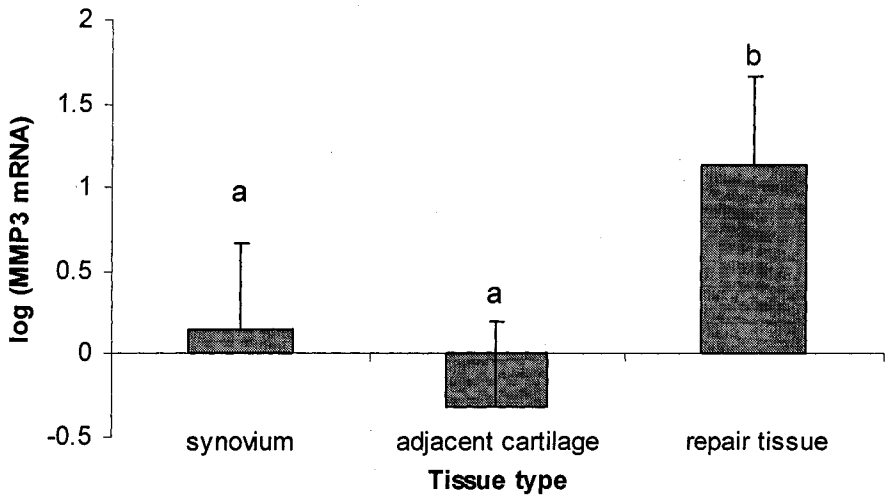

Figure 40 - Log of MMP3 mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

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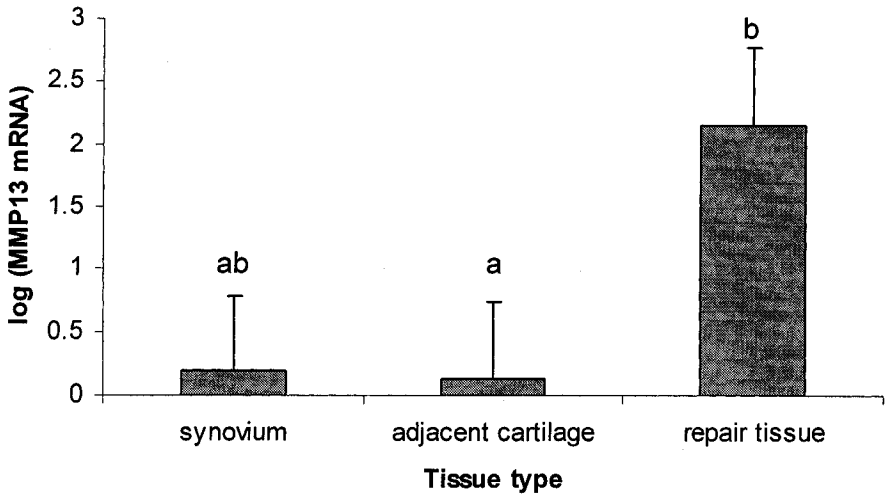

Figure 41 - Log of MMP13 mRNA expression plotted by tissue type. Different letters represent a statistical difference between tissue types (p-value < 0.05).

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9 Endnotes

[i] Clinical pathology services, Veterinary Teaching Hospital, Colorado State University, Fort Collins, CO, USA.

[ii] Quantikine® Human IL-1ra Immunoassay, R & D System, Minneapolis, MN, USA.

[iii] Dynex MRX plate reader, Dynex Technologies Inc., Chantilly, VA, USA.

[iv] C2 ethyl minicolumn, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA.

[v] Savant Speed Vac Plus SC110A, Savant instrument Inc, Holbrook, NY, USA.

[vi] High Sensitivity Prostaglandin E2 Enzyme Immunoassay Kit, Assay Design, Ann Arbor, MI, USA.

[vii] OCT compound, Lab-Tek, Miles Laboratories, Elkhart, IN, USA.

[viii] Olympus IX70 inverted system microscope, Olympus America Inc., Melville NY, USA

[ix] Bioquant 98, Bioquant Image Analysis Corporation, Nashville, TN, USA

[x] Nexes IHC, Ventana Medical Systems, Tucson, AZ, USA

[xi] Ventena Basic DAB Detection Kit, Ventana Medical Systems, Tucson, AZ, USA

[xii] Aggrecan antibody AN9P1, Joint Diseases Laboratory, Montreal, Canada.

[xiii] Type II collagen II-II6B3 antibody, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA.

[xiv] Type I collagen BYA65201 antibody, Accurate Chemical and Scientific Corporation, Westbury, NY, USA.

[xv] S100 antibody Z 0311, Dako, Carpinteria CA, USA.

[xvi] Leica CM 3050, Leica Microsystems, Bannockburn, IL, USA.

[xvii] ABI 7000 Sequence Detection System, Applied Biosystems, Fostercity, CA, USA.

[xviii] Christian M. Leutenegger, Lucy Whittier Molecular Core Facility TaqMan (R) Service Department of Medicine and Epidemiology, School of Veterinary Medicine University of California, Davis, Davis, CA 95616

[xix] Applied Biosystem, Fostercity, CA, USA

[xx] Cyclone Virtishear Homogenizer, Virtis, Gardiner, NY, USA.

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[xxi] Nuclease-Free water, Ambion, Austin, TX, USA.

[xxii] Taqman 96 well plate, Applied Biosystems, Fostercity, CA, USA.

[xxiii] SAS/STAT software, 8.1 edition, 2000, SAS Institute, Cary, NC, USA.

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