

DISSERTATION

**EFFECTS OF EXERCISE ON SYNOVIAL FLUID AND SERUM
BIOMARKERS OF MUSCULOSKELETAL DISEASES IN HORSES
WITH AND WITHOUT OSTEOCHONDRAL FRAGMENTATION**

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2003

UMI Number: 3092648

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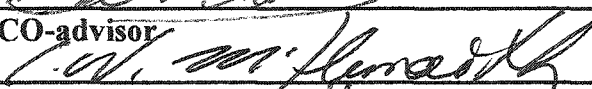
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ABSTRACT OF DISSERTATION

EFFECTS OF EXERCISE ON SYNOVIAL FLUID AND SERUM BIOMARKERS OF MUSCULOSKELETAL DISEASES IN HORSES WITH AND WITHOUT OSTEOCHONDRAL FRAGMENTATION

Introduction: Osteoarthritis (OA) is a common problem in horses.

Measuring biomarkers (BM) is one of the recent methods used to diagnose early OA. With OA, the levels of many BMs usually increase. In addition, many of the BMs are normally increased in body fluid with exercise.

Purpose: To distinguish between changes occurring in osteochondral tissues as a result of adaptation (exercise) versus pathological conditions (osteoarthritis) based on assessing various BMs of articular cartilage and bone metabolism in the synovial fluids and sera of two-year old horses. The changes in the levels of BMs will be correlated with the results of clinical examinations and histopathology findings.

Material and Methods: Sixteen healthy 2-year-old horses were exercised on a treadmill for 91 days (5 days/week). On day 21, an osteochondral chip fragment was created in one randomly chosen intercarpal joint of eight

horses. Both synovial fluid and serum samples were collected weekly from the horses. Bone and articular cartilage BMs were assessed in synovial fluid and serum. On day 91, the horses were euthanized and the pathologic changes in the joints of exercised horses with and without osteochondral fragmentation were scored and then correlated with the synovial fluid and serum BM levels.

Results: Exercise and OA significantly increased all BMs compared to baseline. In general, the increase in the levels of biomarkers was higher as a result of OA compared to exercise. This increase was seen in all BMs except serum CTXI and 234CEQ. The levels of bone markers were higher in serum than synovial fluid samples whereas the levels of articular cartilage markers were higher in synovial fluid than in serum samples. In OA, cartilage metabolism preceded bone metabolism based on BM results. Within cartilage, aggrecan turnover preceded collagen turnover. Moreover, aggrecan synthesis preceded its turnover. The first change in the biomarker levels was in CS846. There were significant correlations between articular cartilage BMs and the clinical examination, gross, and histologic changes of articular cartilage.

Conclusion: This study showed the usefulness of assessing BMs in differentiating exercise-induced changes from discrete OA pathology. Synovitis due to exercise and/or OA may be the key factor influencing the levels of biomarkers. Assessing articular cartilage biomarkers is a useful

**method that can be used to detect the amount of pathological changes
occurred in early OA.**

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ACKNOWLEDGEMENTS

First, I thank Almighty GOD, the Magnificent, the Merciful, who enabled me to write this dissertation successfully. I would like to acknowledge princes Ahmed bin Salman bin AbdulAziz and Sultan bin Mohamed bin Saud who financially supported this study. I am very grateful to my graduate committee, Doctors RC Billinghamurst, DD Frisbie, CE Kawcak, CW McIlwraith, and RD Park for their help, guidance and careful review and valuable suggestions on my dissertation.

My most sincere appreciation is offered to the many people who assisted me in this study. Special thanks are given to Ahmed Al-Othman, Saleh Al-Hamadi, Andi Hume, Megan Knowlton, Heather Colhoun, Claire Miller, Jeruesha Johnson, and to all the volunteers who assisted in this study. I would like also to thank Drs PL Chapman, AH Al-ghamedi, and Richard McNally for their help with statistics.

I am indebted to my country, Saudi Arabia, with emphasis to King Saud University for providing this opportunity and the financial support to complete my graduate study.

Finally, I would particularly like to thank my wife and my daughter whose understanding and patience was a source of great strength and

**encouragement for me in this endeavor. I dedicate this work to my father,
brothers, sisters, wife and daughter.**

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1 THE INTRODUCTION

1.1 The Purpose Of The Study

The purpose of this study is to distinguish between changes occurring in osteochondral tissues as a result of adaptation (exercise) versus pathological conditions (e.g. osteoarthritis) based on assessing various biomarkers of articular cartilage and bone metabolism in the synovial fluids and sera of two-year old horses. The changes in the levels of biomarkers will be correlated with the results of clinical examinations and histopathology findings.

1.2 Specific Objectives

The objectives of this study are:

- 1. To assess the biomarkers of bone and cartilage metabolism in synovial fluids of exercised horses with and without osteochondral fragmentation.**
- 2. To assess the biomarkers of bone and cartilage metabolism in sera of exercised horses with and without osteochondral fragmentation.**

3. To correlate the synovial fluid and serum levels of the bone and cartilage biomarkers with clinical examinations and histopathologic findings.

1.3 Hypotheses

1. In exercising horses, the concentration of synovial fluid and serum biomarkers of articular tissue metabolism will be significantly higher with the presence of osteochondral fragmentation than with exercise alone.
2. Changes in the levels of biomarkers of cartilage and bone metabolism are related to changes detected at clinical examinations and to histopathologic findings.

1.4 Literature Review

1.4.1 Anatomy of Synovial Joints

1.4.1.1 Joint capsule and ligaments

A synovial joint contains the articulating surfaces of at least two bones covered with a layer of hyaline cartilage. The joint is surrounded and stabilized by a joint capsule and ligaments. The joint capsule is composed of two layers: a thick external fibrous layer and a thin synovium that produces, and is in contact with, the synovial fluid contained within the joint cavity. The fibrous layer varies in thickness according to the amount of stress that it supports. It is composed of dense fibrous connective tissue. The collateral ligaments are intracapsular ligaments that often extend between the upper

and the lower bones of the joint. In general, two collateral ligaments (lateral and medial) are present in each joint. Intraarticular ligaments such as the cruciate ligaments in the femorotibial joints and the palmar intercarpal ligaments in the middle carpal joint are short ligaments present inside the joint cavity of various specific joints.

The predominant extracellular components of the joint capsule and ligaments include collagens, proteoglycans, noncollagenous proteins and water. On a dry weight basis, the collagens represent approximately 85% of the total weight of ligaments. Type I is the predominant collagen (Amiel et al, 1984) but types III and V collagens are present in smaller quantities.

The functions of the fibrous joint capsule, collateral ligaments, and intracapsular ligaments are to seal the joint space, provide stability by limiting movements, and provide joint shock absorption. When the fibrous joint capsule and/or ligaments are injured, luxation, subluxation, constriction and/or adhesion to the surrounding structures might occur, which will negatively affect the adaptive function of the capsule and lead to restricted joint movement.

The synovium (also known as the synovial membrane or synovial lining) is the inner layer of the joint capsule. Grossly, normal equine synovial membrane is white to yellowish-white in color (McIlwraith, 1987b). In some regions of the joint, it is smooth and glistening, and in others it contains numerous villi. These villi project inside the joint space and have a different morphology in specific locations (McIlwraith and Fessler, 1978).

Histologically, the synovium is a modified mesenchymal tissue. It consists of two layers called the intima and subintima. The intimal layer lies next to the joint cavity and consists of an incomplete layer (1 to 4 cells thick) with no basement membrane. Two types of synovial cells (synoviocytes) have been identified in this layer: type A and type B. Type A synoviocytes look like macrophages and function as phagocytes and pinocytes (McIlwraith, 1987b). Type B synovial cells resemble fibroblasts, and are thought to be responsible for hyaluronan secretion (Henderson and Pettipher, 1985). The subintimal layer is located deep to the intimal layer, and contains fibrous, areolar, or adipose connective tissues. Fibrous subintima tends to be found in regions where the synovium is subjected to increased pressures. Areolar subintima is found in regions where the synovial membrane moves freely and independently of the fibrous capsule. The subintimal layer has blood vessels that extend within 5-10 μm of the intimal surface. The three principal functions of the synovium include phagocytosis, regeneration, and regulation of protein and hyaluronan content of the synovial fluid by acting as a semipermeable membrane that controls the composition of joint fluid (Van Sickle et al, 1978).

Inflammation of synovial membrane, or synovitis, is often seen with many joint diseases in horses. Arthroscopy and light microscopy of biopsy specimens are two devices routinely used to evaluate gross and histologic structure of synovial membrane, respectively. Synovitis, especially the acute stage, is usually accompanied by some degree of hyperemia, edema and

fibrin deposition. With synovitis, an activated synoviocyte synthesizes and releases substances that can in turn degrade the osteochondral tissues (Robinson et al, 1976; Martel and Pelletier, 1987). Therefore, the synovial membrane can play an important role in the pathogenesis of various arthritic diseases such as osteoarthritis (OA).

1.4.1.2 Osteochondral Tissues

The joint surface consists of a group of layers called osteochondral tissues. Osteochondral tissues consist of (from the most superficial to the deepest layer) articular cartilage, calcified cartilage and subchondral bone.

Articular cartilage covers the articulating surface of the bone. In horses, the normal gross appearance of articular cartilage is translucent and glasslike in the thinner regions, and milky and opaque in the thicker regions (McIlwraith, 1987b). Arthroscopy is the technique routinely used to evaluate the gross changes in the articular cartilage of live horses. With the arthroscope, changes in articular cartilage such as fibrillation, partial-thickness and full-thickness erosions, and wear lines can be easily diagnosed.

In horses, the normal histological structure of articular cartilage is subdivided into four zones: the superficial, the intermediate, the deep and the calcified cartilage zones (McIlwraith, 1987b). The superficial zone (also called the tangential layer) contains small flattened or ovoid chondrocytes and collagen fibrils oriented parallel to the surface of the cartilage. The intermediate zone (or the transitional layer) contains either single or paired, large, rounded chondrocytes and the collagen fibrils are oriented randomly.

The deep zone (also known as the radiate layer) contains large chondrocytes arranged in vertical columns and separated by collagen fibrils that also have a vertical columnar arrangement. The tidemark is a basophilic-staining, undulating line that delineates the elastic, unmineralized zones of the articular cartilage from the zone of calcified cartilage that relatively lacks resiliency. The superficial, the intermediate, and the deep zones are all unmineralized zones. The calcified cartilage (mineralized) zone contains mineralized cartilage and degenerated chondrocytes. It provides a layer of intermediate stiffness between the articular cartilage and the subchondral bone and thus, it anchors the articular cartilage to the subchondral bone. Calcified cartilage modulates the transfer of forces through articular cartilage to bone (Oegema et al, 1997). Therefore it plays a role in converting shear forces in the joint to compressive and tensile forces in the underlying bone (Burr and Radin, 1990). The ratio of thickness of articular cartilage to calcified cartilage in normal joints is approximately 10: 1 (Muller-Gerbl et al, 1987). The elastic modulus of calcified cartilage is more than 10 times lower than that of subchondral bone (Mente and Lewis, 1992). In a recent Study, Murray et al (1999) reported that high intensity exercise led to increased thickness of the calcified cartilage layer without an effect on the thickness of hyaline articular cartilage, and this increase remarkably occurred at sites that withstand high, intermitant loads (Murray et al, 1999). Thus, the calcified cartilage thickness might precede and later lead to the articular cartilage and /or subchondral bone stiffness.

Articular cartilage is composed of cells (chondrocytes) and an extracellular matrix (ECM) that contains collagens, proteoglycans and noncollagenous proteins. Ninety percent of the total collagen in the ECM of hyaline cartilage is type II collagen, which gives the articular cartilage its tensile strength and controls tissue swelling. Other collagens including types VI, IX, XI, XII, and XIV are also present in small amounts in the ECM of articular cartilage. The other major component of the articular cartilage ECM is a proteoglycan that consists of glycosaminoglycan chains covalently attached to a protein core. Matrical proteoglycans have been classified into large (aggregating) and small (nonaggregating) proteoglycans (Heinegard et al, 1985). Aggrekan contains sulfated glycosaminoglycans (sGAGs) including chondroitin sulfate and keratan sulfate that are covalently attached to a protein core. The large aggregating proteoglycan (aggrekan) is formed when many proteoglycan monomers non-covalently attach to the hyaluronan molecule by link proteins. The large aggregating proteoglycan (aggrekan) is the major proteoglycan found in the articular cartilage and its function is to provide the cartilage with its mechanical stiffness and resistance to compression. Proteoglycan can achieve this function because of the negatively charged sGAGs that attract water and cause swelling within the tissue. Several small (nonaggregating) proteoglycans such as decorin and biglycan are also found in the cartilage ECM. It has been reported that the concentration of collagen fibrils is highest in the superficial layer and then sharply decreases with increasing distance from the surface, whereas the

proteoglycan concentration changes in an opposite manner to the collagen concentration (Maroudas et al, 1973). Overall, articular cartilage has the capability of withstanding considerable compressive and tensile forces encountered in weight bearing.

The collagen fibers are continuous between the layers of articular cartilage including the calcified cartilage. However, this continuity of collagen fibers has not been demonstrated at the osteochondral junction (Inoue, 1981; Clark & Huber, 1990). Therefore, it has been suggested that the osteochondral junction might represent a region of weakness, particularly to shear stress (Burr, 1998).

The third component of osteochondral tissues is the subchondral bone, which refers to the zone that separates the calcified cartilage from the marrow cavity. The subchondral bone is subdivided into a thin cortical lamella or subchondral bone plate and trabecular bone. The subchondral bone is composed of a solid mineralized ECM with small canals (canaliculi), spaces (lacunae), and bone cells (osteocytes). Bone material consists of water, mineral, collagen, noncollagenous proteins, lipids, vascular elements, and cells. The mineral is an important structure of bone and is present in bone in a hydroxyapatite form. The second most abundant component of bone is collagen. Type I collagen is the major form in the ECM of bone, accounting for approximately 90% of the total collagen. Noncollagenous proteins represent 5% of the dry weight of bone and lipids account for 2-8% of the organic matrix of bone. In general, the mineral content and collagen

network are both important determinants of the mechanical properties of subchondral bone and how it functions in the body.

The subchondral bone supports the articular cartilage by forming a cushion for the joint by absorbing stress, and forming incongruent joint surfaces that contact on outer surfaces and are separated in the center of the joint (Radin and Paul, 1970, 1971a,b; Kawcak et al, 2001). In addition, the subchondral bone supplies the deeper layer (calcified cartilage) of articular cartilage with nutrients. The subchondral structures and their function are affected by several conditions including physical activity and joint diseases.

Exercise causes an increase in the magnitude of stress to the joint surfaces, particularly in the weight-bearing regions. However a recent study has shown that a low amount of exercise is essential for the normal development of the subchondral bone of young foals up to 5 months of age (Brama et al, 2002). This low level of exercise is very important for the formation of a high quality collagen network and proper mineral content in the ECM of the subchondral bone. This is important for the strength of the subchondral bone (Brama et al, 2002). High levels of exercise usually cause even greater increases in the magnitude of stress on joint surfaces, particularly in the weight-bearing regions. The adaptive method that can be used to oppose stress is an increase in the remodeling rate of the subchondral bone during development. In laboratory animals, remodeling of the subchondral bone results in calcification of the deeper articular cartilage layers (increase bone formation) in an attempt to increase strength and

reduce joint stresses in overload regions (Oegema et al, 1997). This results in a decrease in the normal thickness of articular cartilage and an increase in the subchondral bone thickness with advancement of the tidemark (Radin et al, 1995). This remodeling in calcified cartilage and subchondral bone is demonstrated by increased vascular invasion, increased mineralization around the vascular channels, increased calcified cartilage thickness, and duplication of the tidemark (Radin et al, 1995).

Pool and Meagher (1990) have shown that the subchondral bone of racehorses becomes denser in response to training. The adaptive increase in density of subchondral bone is most evident in the weight bearing regions of the joints (Riggs and Boyde, 1999; Riggs et al, 1999). This is the way that the skeleton adapts to the forces of training and racing (Young et al, 1988) as seen in the middle carpal joints, where Young et al (1991) found that there was an adaptive increase in subchondral bone density in an area approximately 10 mm from the dorsal margin of the radial facet of the third carpal bone. The intensity of exercise also has a relationship with injury in racehorses (Estberg et al, 1996). Musculoskeletal injury is more likely to be seen in Thoroughbred racehorses that run for long distances at high speeds or that rapidly achieve high speeds over a short period of time, than in those at lower exercise intensities (Estberg et al, 1996). It has been thought that remodeling can be driven by bone strain (Frost, 1990), which is based on the magnitude, rate and duration of load on bone (Kawcak et al, 2001). Although it is unknown how the strain induces bone changes, some studies

have shown that bone matrix microdamage experimentally stimulated bone remodeling (Mori and Burr, 1993; Burr and Schaffler, 1997). Osteocytes may also have a role in the response of bone to strain by sensing changes in subchondral bone due to strain and by stimulating bone formation (Duncan and Hruska, 1994). However, it has been found that there was an increase in the numbers of nonviable osteocytes (apoptosis) around the experimentally induced bone microdamage, which later led to increased bone remodeling (Qiu et al, 1997). Thus, it may be concluded that exercise increases loading on weight bearing regions of the joints and this leads to an increase in strain on the subchondral bone. Bone matrix microdamage is the sequela to high stress, which may lead to apoptosis of osteocytes that in turn stimulates bone remodeling in the defect area(s) of subchondral bone.

Because of various amounts of weight bearing applied to the limbs, the forelimbs are under higher stress than the hindlimbs. Therefore, subchondral bone density in the forelimbs might be greater than that in the hindlimbs. Firth (1997) has reported that remodeling of both the radial and third carpal bones occurred in young Thoroughbred horses undergoing a treadmill-training program. On the other hand, Whitton et al (1999) have studied the effect of treadmill exercise on bone remodeling of third and central tarsal bones using MRI and found that there was no significant difference in percentage areas of dense subchondral bone between the trained and untrained horses. The amount of weight bearing and stress magnitudes differ from one site or one bone to another in the same joint.

The radial and third carpal bones in the carpal joint (Firth, 1997) and the third metacarpal condyles in the fetlock joint of a horse (Kawcak et al, 2000) are the sites identified in the forelimb that show an increase in subchondral bone density with exercise. In hindlimbs, it has been reported that the subchondral bone density in the dorsolateral subchondral bone of the central and third tarsal bones was significantly greater than that in the dorsomedial site of these bones (Whitton et al, 1999).

1.4.1.3 Synovial fluid

Synovial fluid represents one of the important components of the synovial joints. It is a modified dialysate of plasma that has the same concentration of small ions and molecules as in plasma, but lower levels of protein (Simkin, 1991). This is because the joint barrier which includes the synovial capillary fenestrations, the intercellular space in the intimal layer and the hyaluronan layer, prevents the large proteins in plasma from entering the joint. Hyaluronan produced by the type B cells of the synovial membrane is added to the joint fluid (Ghadially, 1978). The two major functions of synovial fluid are supplying nutrition to the articular cartilage and limiting friction and wear between opposing articular cartilage surfaces by acting as a lubricant for the articular cartilage and synovial membrane (Mahaffey, 1992).

Synovial fluid examination is often performed for diagnosing some arthritic diseases. It can be used to diagnose and classify various equine joint problems (Persson, 1971; McIlwraith, 1980). Generally, four major

examinations can be performed on the synovial fluid: gross, microscopic, microbiological and biochemical examinations. Gross examination includes the evaluation of volume, viscosity, color and clarity of synovial fluid. Microscopic examination involves a total count of nucleated cells, a differential nucleated cell count and identification of other pathologic materials such as crystals. Microbiological examinations include laboratory procedures used to identify the type of bacteria (such as Gram stains and culture) in septic arthritis. Biochemical examinations consist of several tests, such as the determination of total protein content and the levels of biomarkers of bone, articular cartilage, and synovium metabolism and inflammation.

In the normal situation, there is a balance between the synthesis and the degradation of ECM molecules in articular cartilage and bone. Thus, relatively constant levels of synthesis and degradation products of the matrix are present in the synovial fluids. However in various types of arthritic diseases like osteoarthritis (OA), an alteration in this normal balance occurs, and this leads to changes (increase or decrease) in the release of these products of matrix metabolism into the synovial fluids. The concentration of some of these products can then be measured in body fluids and used as a potential diagnostic tool (biomarkers) in detecting abnormal changes in the joint. Details about specific markers will be described in more detail in the following pages.

1.4.2 Musculoskeletal Diseases and Osteoarthritis in Horses

Musculoskeletal diseases of the equine athlete are a major clinical problem with consequences ranging from lost training time to catastrophic injury and loss of life (Mohammed et al, 1991; Johnson, 1993; Wilson et al, 1993). For example, the economic and emotional impact of fractures in the horse is far reaching, not only in the racing industry but also among companion animals and for their owners. The efficiency of horse performance is based on the integrity and full maturation of the musculoskeletal system. When immature horses are trained for racing, bone resorption is observed resulting in a decrease in bone density (Nielsen et al, 1995). Unfortunately, many horse trainers begin speed training with their horses during this resorption period and before the subsequent remodeling of the bone starts. For this reason, many two-year old horses develop musculoskeletal diseases, such as fractures during their training.

Osteoarthritis (OA) is a common problem affecting horses (Genovese, 1983; Rossdale et al, 1985; Todhunter and Lust, 1990; McIlwraith, 1996). OA is actually a group of disorders characterized primarily by degradation of the articular cartilage accompanied by changes in the bone (osteophyte formation and subchondral bone sclerosis), soft tissues (inflammation), and synovial fluid of the joint. There is limited information available on the natural course of OA. In general, OA may either lead to rapid deterioration of joint integrity or appear as a fairly chronic, stable condition.

The normal biomechanical properties of the joint can be influenced by several factors that may then enhance the development of OA in weight bearing joints. Examples of these factors are congenital and developmental deformities, joint trauma, and certain repetitive loading patterns. As mentioned above, alterations in the normal balance between synthesis and degradation of the articular cartilage and/or subchondral bone are considered important indicators of OA. It is unknown whether this alteration occurs as a result of an inadequate cellular response to the normal articular cartilage and subchondral bone demands, or is due to the insufficient cellular response to supranormal demand from mechanical loading or injury. Both the metabolic disturbances and repair responses of the joint lead to an increase (or decrease) in the release of catabolic and anabolic products in the synovial fluid and later in the systemic circulation and the urine. Measuring the concentrations of these products can give valuable information about the presence and progression of the disease.

McIlwraith (1996) has classified OA in horses into three types. Type I is associated with synovitis and capsulitis, and it is common in high motion joints including the carpal, tarsocrural, and fetlock joints. Type II is associated with (and usually secondary to) other identified injuries or problems including intra-articular fractures, traumatic articular cartilage, ligamentous injuries, osteochondrosis, subchondral bone injury, subchondral cystic lesions, septic arthritis, and fragmentation of distal patella. Type III is incidental or nonprogressive articular cartilage erosion. Since the joint is

considered an interactively functioning unit, all tissues (articular cartilage, synovial membrane, and subchondral bone) will be affected to some extent with OA. Some joints with OA are in active phase, whereas others are inactive or stable (Dieppe et al, 1993). Within the active phase, further destruction of articular cartilage occurs which then leads to metabolically abnormal subchondral bone that can be seen in radiographic images as subchondral bone sclerosis and joint space narrowing (Park et al, 1996).

The main differences between OA in horses and humans is that equine OA can be seen in young as well as old horses whereas human OA is mainly considered a disease of aging. Inflammation is an important feature preceding the occurrence of equine OA, whereas inflammation in human OA is considered as late secondary changes (McIlwraith, 1996). In humans, as well as dogs and other small species, joint instability is considered a significant cause of OA, and thus controlling joint displacement in those species is a major way of controlling the disease. This has led some investigators to use instability models like the Pond-Nuki model in dogs to experimentally induce OA (Pond and Nuki, 1973). In contrast, joint instability is rare as a cause of OA in equine joints.

1.4.2.1 The Role of Synovial Membrane

Inflammation of the synovial membrane (synovitis) and/or fibrous capsule (capsulitis) commonly occurs in the joints of athletic horses (McIlwraith and Van Sickle, 1981). It has been thought that primary or direct synovitis and capsulitis are associated with direct trauma, whereas

secondary or indirect synovitis can occur as a result of the release of cartilaginous wear particles like proteoglycans into synovial fluid (McIlwraith and Van Sickle, 1981). McIlwraith (1979) proved that experimentally induced synovitis alone could lead to osteoarthritis in horses. In addition to pain, synovitis can produce various effects in the joint, which in time can lead to OA. These effects include impairment of diffusion across the synovium and/or a disturbance in the metabolism of synoviocytes (Evans, 1992), decrease of shock absorption, and stimulation of the production of matrix degradative enzymes (e.g. matrix metalloproteinases, cathepsins, and plasmin) by synoviocytes and other cells (e.g. chondrocytes) (Spiers et al, 1994). In addition, the levels of some inflammatory mediators (e.g. IL-1, $\text{TNF}\alpha$, and PGE_2) are usually increased with synovitis (Spiers et al, 1994). Synovitis causes effusion in the joint, which negatively affects the metabolism of synovial cells, and may lead to cell damage. When the cells are damaged, matrix degradative enzymes and cytokines can be liberated into the ECM and after they (the enzymes) are activated, start to degrade the cartilage. Joint effusion causes an increase in the intra-articular pressure (IAP), which may lead to a decrease in blood flow to the joint and cause a decrease in the level of oxygen (Levick, 1990). The increase in IAP will also affect the removal of waste products from the joint, having a negative effect on the joint. Joint stability might also be affected with the increase of IAP. This is possibly more obvious in humans and small animals than in horses because they have relatively small joints. Joint inflammation (if it is not treated)

might lead to fibrosis of the joint, which will negatively affect joint motion (redundancy) and stability.

Osteoarthritis causes gross and histopathological changes in the soft tissues of the joint including the synovial membrane (SM) and fibrous capsule (FC). With OA, the FC becomes edematous and congested. If edema persists, fibroblasts will form collagen fibers that fill the soft tissue planes. This leads to a fibrous thickening of the joint capsule and loss of a normal range of motion of the joint. Arthroscopy is a valuable device to grossly evaluate the SM in the joints (McIlwraith and Fessler, 1978). With synovitis, the gross structure of SM may show some degree of hyperemia initially (McIlwraith and Fessler, 1978). Adhesions and increases in the numbers of SM villi with a change in shape of some of these villi are often seen in the severely inflamed joints (McIlwraith and Fessler, 1978; Frisbie et al, 1997). In chronic situations, the villi become thicker and denser as the disease progresses (McIlwraith and Fessler, 1978).

With synovitis, SM shows changes in its histological structure. Varying degrees of inflammatory cell infiltration and cell hyperplasia in the intimal layer are usually seen in the osteoarthritic joints (Frisbie et al, 1997). Changes in subintimal edema, fibrosis and vascularity can also be seen in joints with synovitis (Frisbie et al, 1997).

1.4.2.2 The Role of Articular Cartilage

Articular cartilage degeneration is considered an important indicator of OA (McIlwraith, 1982). With articular cartilage degeneration, an

alteration in the normal balance between synthesis and degradation of the articular cartilage is seen. When there is an increase in the levels of degradative enzymes, the amount of cartilage resorption might exceed the amount of synthesis. The gross changes that can be seen in degenerated articular cartilage are fibrillation, partial or full-thickness erosion, and wear lines in the cartilage. The histological changes are characterized by superficial to full-thickness fibrillation, chondrone formation, chondrocyte necrosis, and focal loss of chondrocytes (Frisbie et al, 1997).

Biochemically, the content of proteoglycan in osteoarthritic articular cartilage is decreased in time with changes seen in its structure (McDevitt and Muir, 1976; Sweet et al, 1977). This produces some degree of softness in articular cartilage compressability. An alteration in collagen structure has also been reported in articular cartilage with OA (Dodge and Poole, 1989) that causes losses in tensile strength (Kempson et al, 1973). As mentioned previously, the two main matrix molecules of articular cartilage are proteoglycans and collagen (mostly type II). Proteoglycans provide compressive stiffness to the articular cartilage and the collagen network provides tensile strength to the cartilage and controls tissue swelling. The proteoglycans contain negatively charged chains of sGAGs that are very attractive to water and cause swelling of the tissue. The collagen network controls the degree of swelling in the tissue. There is controversy as to whether proteoglycan depletion or breakdown of the collagen framework is the primary pathologic event occurring in early OA. Several researchers

suggest that impairment of the collagen network precedes the degradation of proteoglycans in OA. This hypothesis has been supported by the increase of water content observed in early OA (Maroudas, 1976; Venn and Maroudas, 1977; Weightman and Kempson, 1979). Roberts et al (1986) have found that there was a significant swelling of cartilage with increased compressability and decreased tensile strength but with normal proteoglycan content in articular cartilage with early OA. The impairment in the collagen network is not necessarily the result of alteration in the structure of the type II collagen fibers themselves, but it might be the linking between the adjacent type II collagens by other collagen types (e.g. type IX) and by some other components in ECM (e.g. biglycans).

Other researchers have proposed that enzymatic degradation of articular cartilage precedes morphologic breakdown (McIlwraith and Van Sickle, 1981; McIlwraith, 1982). Proteoglycans are rapidly degraded whereas the turnover of type II collagen is slow. This leads to the second hypothesis, which suggests that proteoglycan depletion precedes collagen breakdown. Several studies have supported this hypothesis (Meachim et al, 1965; McDevitt and Muir, 1976; McIlwraith and Van Sickle, 1981). The degradation of proteoglycans is considered to be a reversible condition (i.e. the cartilage can return back to its normal structure with treatment). However when the degradation of collagen fibrils occurs, this is considered to be irreversible condition, which will not heal with similar structure. Instead, fibrous and fibrocartilagenous tissues replace the normal hyaline cartilage.

Most degradative enzymes that have been investigated in humans and animals are the proteinases. It has been suggested that increased levels of destructive proteinases may lead to degradation of the ECM in osteoarthritic cartilage (Yamada et al, 1987). Of these enzymes, matrix metalloproteinases (MMPs) and aggrecanases appear to play a major role in the degradation of the ECM (Poole, 1990; Sanday et al, 1992; Nagase and Woessner, 1993; Lark et al, 1995; Kaushal and Shah, 2000). Thus, many recent studies have concentrated on producing pharmacological drugs that target these proteinases in the treatment of joint diseases.

The matrix metalloproteinase (MMP) family is a group of zinc-dependent enzymes involved in both normal physiological turnover and pathological degradation of the ECM of various tissues. These enzymes cause degradation of the main components of articular cartilage including, collagens (collagenases) and aggrecans (stromelysins), which may lead to OA. MMPs are produced by a variety of cell types in the body including the chondrocytes, osteoblasts, and synoviocytes in the joint. MMPs can also be released from other cells such as neutrophils, monocytes and fibroblasts (Clegg et al, 1997). They are released as inactive proenzymes. After their release, MMP proenzymes are believed to be activated in the ECM by other degradative enzymes including plasmin and activated MMPs.

Aggrecanases are proteolytic enzymes that have been recently identified as key enzymes cleaving the aggrecan in ECM (Sandy et al, 1992; Lark et al, 1995). At present, two types of aggrecanases have been identified:

aggrecanase 1 and 2 (ADAMTS-4 and ADAMTS-11, respectively). Thus, both MMPs and aggrecanases are potentially key mediators in articular cartilage degradation.

The role of the calcified cartilage and advancement of the tidemark in OA have been studied. Calcified cartilage provides a layer of intermediate stiffness between the articular cartilage and the subchondral bone. The tidemark represents the structure between the calcified and non-calcified cartilage, whereas the osteochondral junction is the junction between the calcified cartilage and the subchondral bone. Tidemark advancement leads to thinning of the overlying articular cartilage (Radin et al, 1995). The concurrent advancement or thickening of the subchondral bone leads to an increase in the thickness of the calcified cartilage zone, and that compromises the normal proportionality between the articular noncalcified and calcified cartilage (Radin et al, 1995). Tidemark advancement can increase mechanical stress over the articular cartilage, which might lead to cartilage loss and OA. It has been found that there is an association between tidemark advancement and cartilage fibrillation (Green et al, 1970; Hulth, 1993).

1.4.2.3 The Role of Subchondral Bone

The loss of articular cartilage that occurs in OA is thought to be partially a result of subchondral bone changes. Several studies have studied the role of subchondral bone in the occurrence of OA (Radin et al, 1995; Burr et al, 1997; Norrдин et al, 1998; Kawcak et al, 2000, 2001). Increases in repetitive impulsive loading in humans are thought to cause microdamage in

the calcified cartilage and subchondral bone, which leads to increased bone density (Radin et al, 1990). In horses, the reason for this increase in bone density is to withstand the forces of training and racing (Young et al, 1988). The amount of subchondral bone sclerosis that is associated with OA might exceed the adaptation level. It has been hypothesized that thinning of articular cartilage is due to advancement of the tidemark as a result to calcified cartilage and subchondral bone sclerosis (Radin et al, 1995). The calcified cartilage and subchondral bone remodeling have been characterized by increased vascular invasion, increased mineralization around vascular channels, increased thickening of calcified cartilage and duplication of the tidemark (Radin et al, 1995). In addition, the expression of type X collagen in osteoarthritic cartilage is similar to that found in hypertrophic chondrocytes during endochondral ossification and this is an indication of the presence of a secondary center of ossification (Hoyland et al, 1991; and Radin et al, 1995).

The histologic changes seen in subchondral bone with OA might be similar to what was mentioned in the adaptive response, which include tidemark advancement, microdamage, osteocyte apoptosis and/or osteonecrosis. However, the magnitude of these changes might be larger with OA than with an adaptive response.

The carpal and metacarpophalangeal joints are the two major joints that exhibit changes in racehorses ranging from simple osteochondral fragmentation (McIlwraith et al, 1987; Kawcak and McIlwraith, 1994; Colon

et al, 1997) to life-threatening catastrophic fractures that lead to death (Mohammed et al, 1991; Johnson, 1993; Wilson et al, 1993). These are often associated within various degrees of sclerosis within the subchondral bone of these joints. Subchondral bone sclerosis and, consequently, OA are common in young racehorses (O'Brien et al, 1981; DeHaan et al, 1987; Young et al, 1988; Pool and Meagher, 1990). The occurrence of subchondral bone sclerosis in OA follows the same processes as mentioned for adaptation. DeHaan et al (1987) have reported that 90% of horses with complete osteochondral fractures of the third carpal bone show severe sclerosis. It has been hypothesized that intense sclerosis leads to increased stiffness and consequent fracture and articular cartilage damage (Pool and Meagher, 1990). The same authors have also hypothesized that the radial facet of the third carpal bone and palmar aspect of the third metacarpal condyle undergo extreme bone formation to the point that the subchondral bone becomes ischemic and necrotic, resulting in collapse of the overlying articular cartilage (Pool and Meagher, 1990).

The formation of periarticular osteophytes is another lesion that can be seen in joints with OA. They are marginal bone projections that blend with the adjacent cortex and subchondral bone plate. One problem that might result from the formation of osteophytes is the loss of normal joint surface congruity and stability in OA. Joint instability and cartilage degradation might stimulate the formation of periarticular osteophytes (Johnson, 1962; Park et al, 1996). Osteophytes are common in instability

models of OA like the Pond-Nuki model in dogs (Pond and Nuki, 1973).

Other periarticular bone changes are the formation of enthesophytes and bone lysis. Periarticular enthesophytes are periosteal bony lesions that result from tearing or avulsion to the insertion line of the joint capsule, tendon or ligament with ossification occurring at the insertion lines (Johnson, 1962).

1.4.3 Equine Osteoarthritis Models

An equine OA model is an experimental system that mimics, as a whole or in part, naturally occurring clinical OA. Several procedures have been performed to create an OA model in horses. McIlwraith and Van Sickle (1981) used the polyene antibiotic filipin to experimentally induce OA in horses. Multiple injections of 400 µg of filipin dissolved in 1 mL of dimethyl sulfoxide were administered in the intercarpal joint. Joint inflammation initially occurs with this model (synovitis), which may lead to OA. Although this model was used as a model of OA in horses, it has some disadvantages. It has been reported that articular cartilage erosion, which is a major sign of OA, was not a feature of this model (McIlwraith and Van Sickle, 1981). Although many studies have recently shown that OA starts as a bone disease (Radin et al, 1984), this model also showed no significant changes in the subchondral bone. It is difficult to control the amount of damage produced by this drug in the joint. Therefore, injected horses showed some variations in the level and location of damage in the joint.

Some researchers have given a nonsteroidal anti-inflammatory drug (NSAID) such as phenylbutazone to control this problem. Although giving

NSAIDs might relatively control the amount of damage, these drugs may negatively affect the mimicry between this model and the naturally occurring clinical equine OA. Another disadvantage of this model is that many injections need to be administered in a single joint in order to induce OA. This may increase the chance of producing an infectious arthritis. However, this model has some advantages over some other equine OA models. It is relatively easy and inexpensive to perform with no need of surgery. In addition, it has been thought that combining this model with a severe exercise regimen may stimulate the occurrence of articular cartilage erosion (McIlwraith and Van Sickle, 1981). Overall, this model appears to be not useful as an experimental model of OA.

Osteochondral fragmentation of the distal aspect of the radial carpal bone has been shown to be the most commonly affected site in the carpi of horses and it leads to clinical OA if not surgically treated (McIlwraith et al, 1987). This has led some researchers to experimentally create an osteochondral fragment in the distal aspect of the radial carpal bone as a new model of OA in horses (Todhunter et al, 1993; Foland et al, 1994). The ability to use arthroscopy on horses has assisted in development of this model. Todhunter et al (1993) used the arthroscope to create a 2.4 cm X .9 cm fragment in the dorsal aspect of the articular cartilage of the distal radial carpal bone and they removed it to expose subchondral bone bleeding. Six days post-surgery, exercise was started, twice daily, in alternating clockwise and counterclockwise directions. Walking and trotting were at about 0.7

m/sec and 1.6 m/sec, respectively. In the first 48 days post-surgery, the horses were only walked for 30 minutes in the beginning and gradually increased to 60 min on day 28 until day 48. After that, both walking and trotting were applied with the amount of walking gradually decreased while the time of trotting was increased until the end of the study (day 90). This model has some disadvantages including the lack of similarity to natural clinical OA in horses. As mentioned above, the distal aspect of the radial carpal bone is the most commonly affected site in horses and if not surgically treated it will lead to clinical OA in the horse. With this model, the fragment is removed and that means the injury is treated. Therefore, this model is better used as a joint healing model than an OA model (French et al, 1989). Only walking and trotting have been used with this model, and galloping has not been used even though it is the most likely cause of the osteochondral fragmentation seen in the distal radiocarpal and third carpal bones (McIlwraith et al, 1987; Firth et al, 1999).

Foland et al (1994) have reported another model of OA in horses. A 9.5 mm curved osteotome was used to create an osteochondral fragment over the dorsomedial edge of the distal radial carpal bone. The medial edge of the fragment remained attached to the synovial plica, and the proximal edge of the fragment remained attached to the joint capsule. The fragment was manipulated to ascertain that it had been freed from the radial carpal bone. For many reasons, this model has shown much similarity to naturally occurring clinical equine OA than the injection or fragment removal models.

In fact with this model, both articular cartilage and subchondral bone were damaged. Since all horses using this model had the same location and size of fragment, the amount of damage produced by this model was controlled. In this model, the fragment was induced in the most commonly affected area in the horse (McIlwraith et al, 1987). Seventeen days after the surgery, exercise commences consisting of 2 min at trot (8-12mph), 2 min at gallop (25-33mph), and 2 min at trot once a day for 5 days/week. This model, in many ways mimics natural OA. However, it has some disadvantages. OA is considered a progressive disease, but there is not enough progression and inflammation with time when using this model. In fact some fragments may heal in this model. Therefore, some changes needed to be performed on this model to stimulate the progression of osteochondral disease and to create significant inflammation in the equine joints.

Recently, a modified osteochondral fragmentation model has been developed (Frisbie et al, 1997). Similar to what was described by Foland et al (1994), an 8 mm osteotome was used to create the osteochondral fragment on the dorsomedial edge of the distal radial carpal bone. The fragment was also allowed to remain adherent to the joint capsule proximally. In addition, a motorized arthroburr was used to form a 15 mm wide defect bed for the 8 mm wide fragment. To stimulate the pathological process, debris from this procedure was not completely flushed from the joint. It has been reported that this model produced adequate pathological changes to provide an acceptable equine model of traumatic osteoarthritis (Frisbie et al, 1997). In

this model, exercised consisted of running on a high-speed treadmill 5 day/week with the same speed patterns from the beginning until the end of the study. Exercise times in this model were a 2 min trot (10-12 mph), 3 min gallop (20 mph), and 2 min trot. The incidence of osteochondral fragmentation and OA in racehorses is associated with speed and not with walking (Firth et al, 1999). Therefore, this exercise regimen is relatively similar to that seen with the natural occurrence of osteochondral fragmentation and OA on racetracks, and overall this model is considered a useful model for OA in horses.

1.4.4 Assessment of Adaptation and Disease Changes in Joint Structures

In general, three methods can be used to diagnose OA in equine joints. These include clinical evaluation, imaging, and the measurement of biochemical marker levels. The major difference among these methods is how early in the disease process that each can detect pathological changes; ie. their sensitivity.

1.4.4.1 Clinical Examination

Lameness is the most common cause of early retirement in equine athletes (Jeffcott et al, 1982). It can be detected in a clinical examination by watching the horse in motion. In horses, lameness during the trot has been classified into six scores, 0-5 (American Association Equine Practitioners, 1991). In addition, flexion of some suspected joints (e.g. carpus, fetlock, and

tarsus) is performed and the response to flexion has been graded into 5 scores, 0-4 (Frisbie et al, 1997). Effusion refers to an accumulation of synovial fluid within the joint, which reflects a combination of increased fluid production (synovitis) and decreased fluid removal. Effusion can be seen as an adaptive or disease sign. An adaptive effusion is temporary and is usually seen in one or more joints of healthy young racehorses early in their training period. Disease effusion is present within injured joints, and the severity of effusion has been graded at five levels, 0-4 (Frisbie, 1999).

Exercise itself may cause lameness in horses (Todhunter et al, 1993, Kawcak et al, 2000). Kawcak et al (2000) have reported that 4/6 horses became lame approximately three months after the beginning of exercise on the treadmill.

With OA, cartilage erosion occurs and it is probable the degree of lameness increases with an increase in the amount of articular cartilage erosion. It has been reported that pain itself is considered one of the strongest independent predictors of the progression of OA in human osteoarthritic knees (Dieppe et al, 1993).

Although clinical examination is a routine method used to detect lameness in musculoskeletal diseases of horses, this method has some disadvantages. In general, clinical examination is used to diagnose lameness, which often appears in the advanced stages of equine OA. Thus, a clinical examination is often times insensitive for detecting OA in its early stages. In addition, the clinical examination is mainly subjective and may differ from clinician to

clinician. Therefore, new diagnostic methods are required to reliably diagnose OA in its early stages.

1.4.4.2 Imaging Techniques

1.4.4.2.1 Conventional radiography

Radiography is the most widely used imaging technique for the diagnosis of osteochondral diseases. In general, routine radiography is an excellent technique for imaging bone, but it is a poor technique for imaging articular cartilage and soft tissue. Other disadvantages of radiography include its inability to be used to diagnose early changes in bone, and the appearance of overlapping bone in 2-dimensional images (Kawcak, 1998). Therefore, multiple 2-dimensional images should be taken for evaluation of a 3-dimensional structure (Kawcak, 1998). It has been reported that approximately 30-40% change in bone mineral density is required before bone changes can be seen in radiographs (Greenfield, 1986). Because radiographic changes are relatively slow, several months are usually necessary to accurately assess the progression of joint damage or its reduction by treatment.

Minimal radiographic changes can be appreciated in joints as a result of adaptive exercise (Kawcak et al, 2000). Todhunter et al (1993) assessed the effects of postoperative exercise on the repair of osteochondral fragmentation in the intercarpal joints of ponies. Six weeks post exercise, they found a significant increase in the ratio of the soft tissue thickness to the width of the

third carpal bone using the dorsolateral-palmaromedial oblique radiographic view (Todhunter et al, 1993).

With OA, several changes can be detected with radiography (Park et al, 1996). These include the following:

Tissue changes

- a. Increased subchondral bone density
- b. Articular cartilage loss
- c. Bone formation within and around the joint
- d. Tearing of ligaments, tendons, or joint capsule
- e. Bone lysis in sliding or gliding joints (e.g. intertarsal or tarsometatarsal joints)
- f. Joint effusion

Radiographic changes

- Subchondral bone sclerosis
- Joint space narrowing
- Osteophyte formation
- Enthesophyte formation
- Radiolucency in subchondral bone plate
- Expansion soft tissues

Although joint space narrowing and subchondral bone changes are considered key features of OA, different signs of OA can be seen in different cases of OA. In a five-year follow up study in humans, Dieppe et al (1993) have reported that the radiographic features of 120 human osteoarthritic knees included 20% osteophytosis, 18% subchondral bone sclerosis, 4% and 39% joint space narrowing in femoropatellar and femorotibial joints, respectively. The reason for this is unknown but several factors including age, type of job, type of joint, type of OA, and the amount of stress and weight bearing on the joint may play roles in accounting for these differences. Although these radiographic changes are often used to detect OA, they can lead to false interpretation. As mentioned above, the articular

cartilage thinning is radiographically determined by measuring the joint width. However, this method is inaccurate because the joint width can vary with age, standing position during radiography and the type of joint (Adams and Wallace, 1991). It has also been reported that radiographs are insensitive for detecting subchondral bone sclerosis when compared to histologic changes in the tarsus of the horse (Lavery et al, 1991). In addition, overlapping structures may lead to misdiagnosis of sclerosis (Widmar et al, 1994). However in humans, a correlation between clinical examination and radiographic abnormalities was reported in osteoarthritic knees (McCrae et al, 1992).

1.4.4.2.2 Nuclear scintigraphy

Nuclear scintigraphic imaging is based on the functional distribution of a radiopharmaceutical in the body, and it reflects the physiologic activity of the tissues (Park et al, 1996). Three phases can be identified to evaluate the musculoskeletal system. These are the flow (perfusion) phase, the pool (soft tissue) phase, and the bone (late) phase (Park et al, 1996). The flow phase represents radiopharmaceutical in blood vessels and lasts for 1-2 minutes post injection. The pool phase occurs 10-20 minutes after the flow phase and it represents radiopharmaceutical distribution in the extracellular fluid. The bone phase represents radiopharmaceutical distribution in bone and it starts several hours (≥ 2 hours) after the injection. Bone scans image the osteoblastic activity of the bone tissue (Park et al, 1996).

Nuclear scintigraphy has some advantages and disadvantages compared to radiography. Nuclear scintigraphy has been used in humans and animals in diagnosing lesions and evaluating treatment. Many acute bone diseases can be diagnosed by nuclear scintigraphy that cannot be discerned from radiographs until the condition has become chronic. In addition, nuclear scintigraphy can be used to diagnose some vascular and soft tissue diseases. Therefore, nuclear scintigraphy may be able to differentiate bone diseases from soft tissue diseases when mixed lameness is the presenting sign. Another advantage of scintigraphy in horses is that it offers accurate imaging of the upper limbs, pelvis, and vertebral column without the need for general anesthesia. It has been reported that nuclear scintigraphy is a very sensitive method to detect small osteochondral fragments, but it has very low specificity to detect the type of lesion and to differentiate between normal and diseased remodeling of bone (Kawcak et al, 2000). Therefore, another imaging procedure (usually radiography) should be used in combination with this method. That means the anatomic picture of a nuclear medicine image is not as detailed as radiographs and other advanced imaging techniques.

Nuclear scintigraphy has been used subjectively and quantitatively to evaluate the effect of exercise on the subchondral bone of carpal and metacarpophalangeal joints in horses (Kawcak et al, 2000). Nuclear scintigraphy has been performed at the beginning and end of a six-month study on twelve healthy horses that were randomly assigned to either control (hand walked, n = 6) or treatment (treadmill exercised n = 6) groups. For the

subjective evaluation, the intensity of radiopharmaceutical uptake was graded in the carpal bones, proximal sesamoid bones, third metacarpal condyles, and proximodorsal aspect of the first phalanx in the metacarpophalangeal joint. The investigators found there was significantly more radiopharmaceutical uptake in the proximal sesamoid bones and third metacarpal condyles of the fetlock joints in the exercised group compared to the control group. However, no significant differences have been detected in the carpus or first phalanx between groups (Kawcak et al, 2000). The investigators thought that the type of exercise (treadmill and not racetrack) and the duration of exercise might not have been enough to induce carpal bone damage (Kawcak et al, 2000).

Nuclear scintigraphy has been used to assess OA in horses. Generally, increased uptake can be seen in joints with OA. Todhunter et al (1993) used the pool and late phases of nuclear scintigraphy as measurement methods to evaluate the response of equine joints with osteochondral defects to postoperative exercise and intraarticularly administered polysulfated glycosaminoglycan. They found that radionuclide uptake on blood pool images decreased faster than that on delayed images, in which uptake remained high for 17 weeks, and this indicated that bone was metabolically active for at least 17 weeks. Increased uptake during the soft tissue (pool) phase is usually seen in synovitis or capsulitis (Canapp et al, 1999).

The relationships between nuclear scintigraphy and other diagnostic methods have been determined in both humans and animals. Ross (1998)

studied the correlation between bone scintigraphy, radiography and clinical findings in 114 Standardbred horses that showed lameness in the metatarsophalangeal joints. He concluded that the horses with advanced scintigraphic findings were more likely to have lameness and radiographic evidence of subchondral bone damage. A correlation between clinical examination, radiography, and the localization of scintigraphic abnormalities has been also reported in humans with OA (McCrae et al, 1992; Dieppe et al, 1993). Dieppe and co-workers (1993) have reported that there was a positive correlation between bone scan abnormalities and some radiographic features of OA including osteophyte score and the presence of subchondral bone sclerosis in humans. They have found that radiographic variables could only account for 63% of the scan abnormality (Dieppe et al, 1993).

In a cross-sectional study of 100 human patients with osteoarthritic knees, McCrae et al (1992) have identified four abnormal patterns in the bone scan that can be seen in scintigraphic images during the bony phase in osteoarthritic knees in humans: uptake occurred on the joint line (tramline pattern), extended to the subchondral bone (extended pattern), all around the joint (generalized pattern), and isolated to the patella (the hot patella). They concluded that these different patterns of scintigraphic abnormality might reflect the activity in various aspects of the osteoarthritic process. They found that there was strong correlation between the uptakes in the tramline pattern of bone scintigraphy and subchondral bone sclerosis on a radiograph. The extended pattern of isotope uptake in subchondral bone

was associated with more severe radiographic damage, which may indicate an advanced change or active disease progression. There was also a strong correlation between the generalized pattern and both joint pain and osteophyte formation and it is thought that this pattern has the most clinical significance. There was no correlation between the hot patella and the radiographic changes other than cyst formation (McCrae et al, 1992). Therefore, bone scan may reflect the activity of the disease process and not its morphologic consequences. In addition, positive bone scans have also correlated with some clinical findings including pain and stiffness (Dieppe et al, 1993). In humans with osteoarthritic knees, both clinical and radiographic features can achieve a success rate of 76% in prediction of scan abnormalities (Dieppe et al, 1993).

Nuclear bone scintigraphy also has been used to predict five-year subsequent outcomes of OA in human knees (Dieppe et al, 1993). It has been found that a positive bone scan was a significantly positive predictor of subsequent joint space narrowing, which is an indicator of cartilage destruction in osteoarthritic knees (Dieppe et al, 1993). Bone scans however have more predictive value for changes in the joint space than for changes in the bone itself such as osteophytosis or subchondral bone sclerosis (Dieppe et al, 1993).

The relationships between synovial fluid markers of cartilage and bone turnover and scintigraphic scan abnormalities have been studied in osteoarthritic knees of human patients (Sharif et al, 1995). They found that

there was a positive association between the severity of late-phase bone scan abnormalities and the SF levels of osteocalcin. Mean osteocalcin levels were 47% higher in patients with severely abnormal findings on scans compared with levels in patients with mildly abnormal scan findings. It was concluded that the abnormalities seen in bone scan positively correlate with the synovial fluid biomarkers of bone turnover (Sharif et al, 1995). In addition, insignificant increased trend in KS/sGAG and CS/sGAG ratios in osteoarthritic patients with abnormal bone scan uptakes was noted (Sharif et al, 1995). This was supported by Dieppe et al (1993) who found that a positive bone scan could be used to predict a subsequent joint space narrowing. In a five-year follow up study, it has been reported that the identification of OA progression or the appearance of radiographic changes was seen in 60% of patients that showed bone scan abnormality at entry and normal radiographic imaging (Dieppe et al, 1993).

1.4.4.2.3 Computed Tomography

Computed tomography (CT) is a method of obtaining information from the passage of X-ray beams through a selected area of the body with the aid of a computer. With CT, the image is made with X-rays from a scanner as the X-ray tube travels in a 360° arc. Several direct 3D images at specified slice thicknesses can be produced by the CT technique. CT is an excellent method to detect subchondral bone density and small fragments (Kawcak et al, 2000). Due to image in a third dimensions, CT images show better definition of bone lesions than radiographic images.

Some soft tissue structures in the joint are also better defined with CT. One other advantage of CT over radiography is that the superimposed structures seen in radiographs are not seen with CT. CT has not been routinely applied to horses.

Several studies have used CT to assess bone changes as a result of exercise. Kawcak et al (2000) have reported that six months of exercise on the treadmill resulted in significantly increased mineralization of subchondral bone in the distal third metacarpal condyles, but not in the radiocarpal and third carpal bone surfaces. The reason for these findings was thought to be that the amount and/or the duration of exercise were not enough to produce changes in these bones. In addition, CT osteoabsorptiometry analysis may not be sufficiently sensitive to detect changes in carpal bones. In rats, Forwood and Parker (1991) have reported that exercise led to increased bone formation in the femurs but not in the tibias. Therefore, each bone responds differently to exercise. The amount of weight bearing that each bone can withstand might play a role in the different responses to exercise. In human shoulder and elbow joints, CT osteoabsorptiometry has been used to show that joint surface density is an indicator of loading history in both normal and diseased joints (Muller-Gerbl, 1998).

Riggs and Boyde (1999) used CT to measure the bone density in distal regions of the third metacarpal bones obtained from 12 Thoroughbred horses (six horses with high intensity exercise and six horses with only

walking). They have reported that bone density was greater in specimens from the exercised group than the control group. They proposed that the increase in bone formation might lead to increased stiffening within the condyles. This, in turn, may lead to increased concentration of strain at the condylar grooves with initial cracking through the calcified cartilage and into the subchondral bone. In time, it may lead to the occurrence of OA in the joint. In humans, CT has been used to diagnose osteochondral lesions (Farmer et al, 2001), but this has not reported in horses.

1.4.4.2.4 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a powerful tool for the clinician to diagnose joint injuries in human athletes. It has essentially replaced arthrography for evaluation of disorders within the joint. MRI is able to visualize all components of the joint and is considered the best method to image the articular cartilage and soft tissues within joints (e.g. ligaments and menisci). The problem in applying this technique to veterinary medicine is its high cost and the difficulty in using it on live horses. In addition, there appears to be no completely standard description for each joint using MRI to allow differentiation between the normal and pathologic joints. Even though this is true, many investigators have used MRI to diagnose various musculoskeletal diseases in horses.

Several studies have used MRI to evaluate the effects of exercise on joint structures of horses. MRI has been used to assess subchondral bone density in young Thoroughbred horses as an aim to evaluate the effect of

treadmill training on bone remodeling of third and central tarsal bones (Whitton et al, 1999). Twelve untrained thoroughbred horses were divided into training and walking groups. MRI was used to make sagittal and oblique sagittal imaging slices of the left third and central tarsal bones. Image analysis software was used to measure the proportion of dense subchondral bone in the dorsal facet of each bone. The percentage areas of dense subchondral bone in the dorsal facet of the central and third tarsal bones were measured in both groups. No significant difference was found in percentage area of dense subchondral bone between the two groups (Whitton et al, 1999)

Synovitis and capsulitis are early conditions that can lead to OA in horses (McIlwraith, 1979). Conventional radiography cannot detect soft tissue changes. Although clinical examination (effusion) and synovial fluid analysis (total protein and white blood cell count) can help to detect synovitis, recent studies have shown that MRI is a very sensitive technique to assess joint inflammation and destruction and it is positively correlated with gross changes (Sabiston et al, 1987) and histologic pathology seen in synovium (Sabiston et al, 1987; Østergaard et al, 1997 & 2001). This was achieved by macroscopically and microscopically grading the synovitis of synovium biopsies taken from joints with OA and comparing these grades with the synovial membrane and joint effusion volumes detected by MRI (Østergaard et al, 1997). Therefore, MRI

might be used as a sensitive predictor of early osteoarthritis and a valuable predictor of long-term therapeutic effects in osteoarthritis.

Martinelli et al (1996) have reported that arthroscopy and MRI are the best procedures to evaluate articular cartilage degradation in racehorses (based on a single case study and the MRI was done postmortem). In fact, investigators in one study preferred MRI over arthroscopy and reported that subtle changes in the articular cartilage may produce abnormal MRI changes while looking completely normal through the arthroscope (Reiser et al, 1988). In addition, it has been reported that, with high resolution, MRI was able to demonstrate two and three zones of differing signal intensity in bovine and human articular cartilage, respectively (Lehner et al, 1989; Modl et al, 1991). However since MRI is difficult to use with live horses, arthroscopy is still considered the gold standard for detecting articular cartilage degradation.

MRI has been used to evaluate articular cartilage changes over 3 years in dog knees that had experimentally induced anterior cruciate ligament transections (Braunstein et al, 1990). In addition to osteoarthritic abnormalities usually seen in bone with conventional radiographs, MRI showed changes in articular cartilage thickness, degenerative meniscal tears, fluid-filled subchondral cysts, and the absence of the anterior cruciate ligament (Braunstein et al, 1990). Increases in articular cartilage thickness seen by MRI might indicate that

articular cartilage in OA is capable of undergoing hypertrophic repair resulting in increases of articular cartilage proteoglycan concentration and cartilage bulk (Braunstein et al, 1990). However, the increase in articular cartilage thickness might come from the increase in water content due to an impaired collagen network in early OA (Maroudas, 1976; Venn and Maroudas, 1977; Weightman and Kempson, 1979).

Several studies have analysed the relationship between MRI and other diagnostic techniques. It has been found that MRI reveals more details about joint structure abnormalities than radiography or nuclear scintigraphy (Braunstein et al, 1990; Martinelli et al, 1996; Rodkey et al, 1999). This is an expected result because MRI is considered an excellent technique to evaluate the articular cartilage and other soft tissues within the joint. It has been reported that there was correlation between the scintigraphic patterns including tramline (osteophyte formation), extended (activity in subchondral bone) and patellar with MRI images in osteoarthritic knees (McAlindon et al, 1989).

MRI has been shown to be an excellent technique for diagnosing certain musculoskeletal diseases other than OA in horses. It has been shown that MRI is a much more valuable technique than conventional radiography or CT in diagnosing lameness in the coffin joint of horse (Whitton et al, 1998) and navicular syndrome (Widmer et al, 2000).

1.4.4.3 Biomarkers

Although various imaging modalities have been used to diagnose OA in horse joints, they still have their disadvantages including inability to diagnose early OA (radiography and CT), a lack of specificity (nuclear scintigraphy), or difficulty in application (MRI). Therefore, more specific, sensitive and easily applicable techniques are needed to identify OA in its early stages and to monitor therapeutic response and the healing process. Measuring the levels of biomarkers that reflect abnormalities of the turnover of bone, cartilage, and synovium may be a useful method for the investigation and monitoring of OA.

Musculoskeletal biomarkers are molecules of connective tissue matrices released into biological fluids during the process of tissue turnover. A good biomarker of a tissue should be specific for that tissue (e.g. bone, cartilage, or synovium), reflect either formation or resorption, be stable, have a reliable standard for assay, and be adaptable to a simple, comparatively inexpensive, and automatable assay.

The advantages of using biomarkers over imaging techniques in identifying OA are their ability to diagnose the disease in its early stages and also to monitor its progression due to their faster response in detecting changes compared with imaging (X-rays). Unlike bone scans, biomarkers are not only specific to the tissue but also specific for structures within the tissue. For example in the articular cartilage, there are biomarkers that are specific for type II collagen and others that are specific for aggrecan. In

addition, there are biomarkers that are specific for either tissue synthesis or resorption. The assays used to assess the levels of biomarkers are generally very accurate. Thus, biomarkers can accurately detect OA in its early and reparative stages.

In general, biomarkers of musculoskeletal system can be divided into those of bone, articular cartilage, and synovium metabolism.

1.4.4.3.1 Biomarkers of bone formation

Biomarkers of bone formation can be measured in synovial fluid, serum, or urine. The most commonly assessed bone formation markers are bone specific alkaline phosphatase (BAP), the C-propeptide of type I procollagen (PICP), the N-propeptide of type I procollagen (PINP), and osteocalcin. Each one of these markers reflects a different stage of osteoblastic differentiation. During bone formation, matrix formation precedes matrix mineralization. Since PICP and PINP are released prior to the formation of type I collagen molecules, these propeptides are considered early markers of bone formation. BAP is also an early bone formation marker because it is a cell membrane enzyme found in preosteoblasts and osteoblasts. In contrast, osteocalcin is considered a later marker of osteoblast differentiation because it is thought to be involved in regulating osteoid mineralization (Lian et al, 1982).

1.4.4.3.1.1 Alkaline phosphatase (AP)

Alkaline phosphatase is a membrane-bound protein with enzymatic activity in hydrolyzing phosphate esters. Its mechanism of release and specific function(s) are still unknown. Although total alkaline phosphatase (TAP) is not specific to bone, its levels in serum have shown a correlation with bone formation rate as assessed by calcium kinetics in normal patients (Weaver et al, 1997). It is usually measured by an automated colorimetric method. TAP levels in serum consist of several isoforms of alkaline phosphatase produced by bone, liver, intestine, kidney, spleen and placenta (Moss, 1987). The majority of TAP in serum is the liver and bone isoforms. Therefore, it lacks sensitivity and specificity for measuring bone formation. In fact, the distinction between these two isoforms is important for reliable clinical utility.

The bone-specific alkaline phosphatase (BAP) isoenzyme is the major component of TAP in the serum of young horses. Eighty to ninety percent of TAP in newborn foals is BAP and that decreases markedly over the first 21 days post partum (Hank et al, 1993). In adult horses, only 17.9% of TAP is derived from bone (Hank et al, 1993). BAP can be measured using several techniques including heat inactivation, chemical inhibition, electrophoresis, wheat germ lectin (WGL) precipitation, and immunoassay (Hank et al, 1993; Price et al, 1995, 2001 and Van Hoof & De Broe, 1994). Immunoassays including enzyme immunoassay (EIA) and immunoradiometric assay (IRMA) have been also used to measure the concentration of BAP in humans

and horses (Garnero & Delmas, 1993; Gomez, Ardakani et al, 1995 and Fuller et al, 2001).

It has been reported that the concentration of serum BAP in horses is inversely correlated with age, with the most significant changes during the first two years of life (Price et al, 1995, 2001). BAP has been used to monitor the effect of treadmill exercise on the developing skeleton of two-year-old horses (Price et al 1995). They found a significant decrease in serum BAP in the exercised and control groups compared with the baseline values as a result of age changes. However, when controlled for age, it was found that exercise had a positive effect on the levels of BAP compared to control groups with significant differences in the second and fourth months of exercise. In humans, the levels of BAP were significantly higher in the circulation of exercised young males compared with non-exercised controls (Eliakim et al, 1997). However in a recent study, Kawcak et al (2000) did not detect significant differences in BAP levels between an exercised and a non-exercised control group of horses. The age of horses, length of the study, type of exercise program and the type of BAP assay used in each study were not the same as in the Price study and this may explain the different results in these studies.

With osteoarthritis, the levels of BAP usually change. In a recent study, Fuller et al (2001) studied the levels of biomarkers in the synovial fluid of horses and determined the differences between normal and osteoarthritic joints. They found that there was a significantly higher level of synovial fluid

BAP in osteoarthritic joints compared to normal. In addition, there was a positive correlation between the degree of articular cartilage degradation determined by arthroscope and the levels of synovial fluid BAP, which might suggest a link between bone turnover and cartilage degradation in osteoarthritis (Fuller et al, 2001).

Neither TAP nor BAP have been assessed in the present study. We decided not to assess TAP because it lacks sensitivity and specificity for measuring bone formation. Although BAP is considered a specific marker for bone formation, the cross reactivity with non-skeletal isoenzymes is still an issue (Garnero et al, 1993; Gomez et al, 1995). Moreover, it has been shown that osteocalcin is better correlated with bone formation than BAP (Eastell et al, 1988). In addition, it has been shown that changes in the levels of osteocalcin as a response to treadmill exercise were faster than changes in the levels of BAP (Jackson et al, 1998).

1.4.4.3.1.2 C & N- terminal propeptides of type I procollagen (PICP & PINP)

PICP and PINP are globular extensions found at the C and N termini, respectively, of type I procollagen (collagen precursor) (Figure 1).

Immediately after the osteoblastic release of the type I procollagen, procollagen N-proteinases and C-proteinases cleave PINP and PICP propeptides, respectively, and the collagen molecule is formed. The cleaved propeptides are released and then enter the circulation. PICP and PINP are considered early markers of bone formation. PICP is correlated with mineralization rate as measured by calcium kinetics in normal people

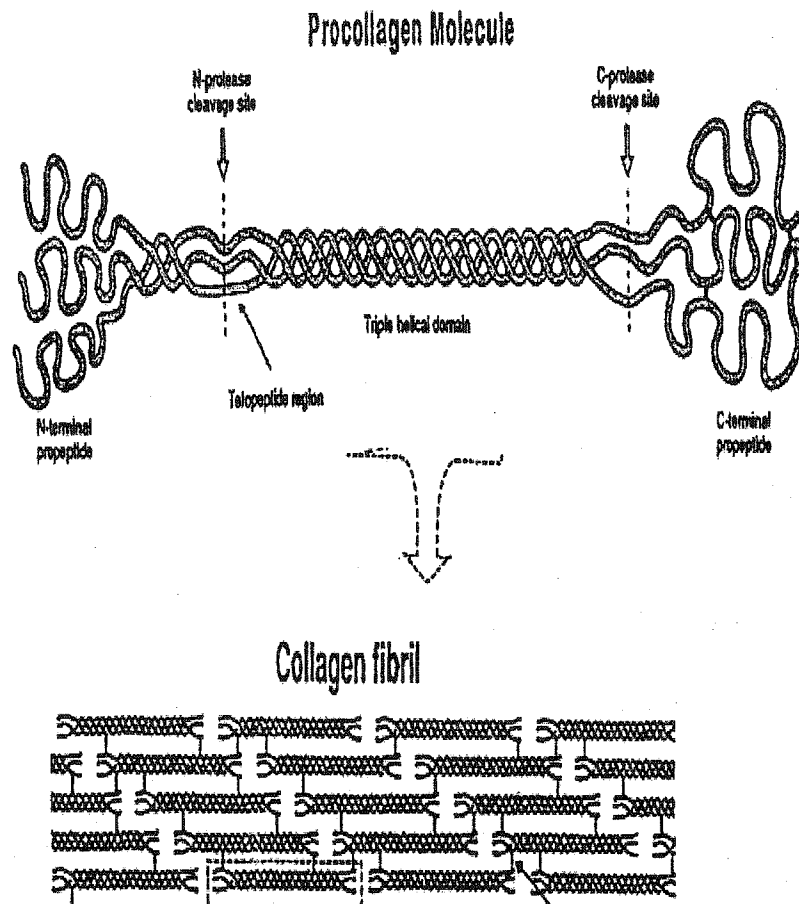


Figure 1. This figure shows C- and N- termini propeptides of type I (PICP and PINP) or type II (CPII and NPII) procollagens.

(Charles et al, 1994). Most studies have measured PICP levels and few have monitored PINP.

The concentrations of PICP and PINP can be measured using various types of immunoassays including radioimmunoassays or ELISA assays (Melkko et al, 1990; Ebeling et al, 1992; Winterbottom et al, 1993; Linkhart et al, 1993; Ørum, 1996). The concentration of PICP is negatively correlated with age, and the most significant changes in horses are seen in the first two years (Price et al, 1995, 2001). In human, levels of serum PICP increase linearly with age in normal adult women, and they decrease with age in normal adult men (Ebeling et al, 1992). On the other hand, the concentrations of serum PINP decrease with age in normal adult women and they do not change with age in adult men (Ebeling et al, 1992).

Exercise may have an effect on the levels of propeptides of type I procollagen. It has been reported that serum concentration of PICP decreased over a one-year study period in both exercised horses and controls due to age (Price et al, 1995). However when controlled for age, the levels of PICP were higher in the exercised group than the control group with a significant increase by four and ten months of age. A significant increase in the levels of PICP during exercise has also been reported in humans (Brahm et al, 1997b; Eliakim et al, 1997; Wallace et al, 2000). In another study, the levels of PICP in serum were lower in human runners than in controls (Brahm et al, 1997a). However in a recent study, Kawcak et al (2000) have found no significant differences in PICP levels between exercised and

controlled group of horses. Similar results have been also reported in humans (Fujimura et al, 1997). This conflict between the studies can be explained by the same reasons mentioned with BAP.

Very few studies that assessed PICP in human with OA have been reported. The concentration of PICP obtained from the serum of patients with varying severity of OA did not significantly differ from the normal controls (Akimoto et al, 1997). So far, this marker has not been evaluated in relation to OA in horses. It has been reported that serum PINP is a more sensitive marker than PICP for detecting osteoporosis in women (Ebeling et al, 1992; Garnero et al, 1997). Although this difference might be truly significant, the sensitivity of the assay used might be responsible for this difference (i.e. the assays used to detect PINP have higher sensitivity than those used to detect PICP).

In this study, we decided not to assess PICP and PINP as markers of bone formation. The main reason for this is because these markers are not specific only for bone. In addition to bone, type I procollagen is present in such tissues as tendons, ligaments, skin, cornea and many interstitial connective tissues. Thus, it is possible that some of the procollagen propeptides present in body fluids comes from non-bony sources. Several researchers have assessed these propeptides as markers of bone formation. Their value as bone markers may be because bone is metabolically active with a higher turnover rate than other soft tissues and theoretically that would imply that PICP and PINP mainly reflect type I bone collagen

formation. The sensitivity of PICP in identifying subtle alterations of bone turnover is questionable especially during phases of rapid bone growth (Blumsohn et al, 1994; Charles et al, 1994). This has led some investigators to conclude that PICP might not be a specific marker of bone formation (Blumsohn et al, 1994). In general, the ELISA for PICP is safer and easier to perform than the radioimmuno-assay, however there is no cross-reactivity between human and equine procollagen propeptides by the antibody used in the PICP ELISA, according to the manufacturer (Metra Biosystems¹). Treadmill exercise causes changes in the level of osteocalcin faster than the changes seen in the level of PICP (Jackson et al, 1998). For all of these reasons, we decided to use osteocalcin and not these propeptide markers as bone formation markers in this study.

1.4.4.3.1.3 Osteocalcin

Ten percent of the organic matrix of bone consists of noncollagenous proteins including osteocalcin, osteonectin, osteopontin, bone sialoprotein, growth factors, serum proteins (albumin and immunoglobulin), and proteoglycans including decorin and biglycan. Most of these molecules are present in other tissues except osteocalcin, which is unique to bone. Osteocalcin, also called gamma carboxy-glutamic acid (Gla) protein or Bone Gla protein (BGP), is a small vitamin K-dependent and calcium-binding protein that contains 49 amino acid residues with three Gla residues. It composes 1-20% of the noncollagenous protein of the organic matrix of bone

depending on species, age, and site (Conn & Termine, 1985; Hauschka, 1979; Lian et al, 1982; and Price, 1983).

Osteocalcin is biosynthesized and secreted by osteoblasts (Beresford et al, 1984 & Lian et al, 1985) and therefore, it is considered a specific osteoblastic marker produced during bone formation. Most of the secreted osteocalcin is deposited in the extracellular matrix of bone and only a small amount enters the blood. The levels of circulating osteocalcin are used as specific markers for osteoblastic activity and bone formation in metabolic bone diseases. The majority of circulating osteocalcin are the intact molecules and large fragments with some small fragments also present (Garnero, 1994; Chen, 1996). The intact osteocalcin (1-49 residues) represents 36% and the large N-terminal mid fragment (1-43 residues) represents 30% of total circulating osteocalcin. Other fragments include the smaller N-terminal (1-19 residue), middle (20-43 residues), and C-terminal (44-49, 20-49 & 43-49 residues) fragments (Garnero et al, 1994). The source of osteocalcin fragments is either from osteoblastic synthesis, catabolism of the intact molecule in bone, or from proteolysis in blood or other tissues (Calvo et al, 1996).

Although the precise biological function of osteocalcin in bone has not been determined yet, some authors suggest that this protein plays a role in the mineralization of bone matrix by regulating the growth of the hydroxyapatite crystals (Hauschka et al, 1975; Hauschka & Gallop, 1977;

Boivin et al, 1990). Therefore, it is considered a later marker of osteoblastic differentiation.

Various methods have been used to measure circulating osteocalcin levels. These include high performance liquid chromatography (HPLC), radioimmunoassay (RIA), immunoradiometric assay (IRMA), enzyme linked immunosorbent assay (ELISA), and luminescence immunoassay (LIA) (Power and Fottrell, 1991). In most of these assays, the osteocalcin antibodies have been made against the intact molecule (human or bovine). However, the antibodies can also react with the various fragments of osteocalcin found in the circulation.

Sex and age have an effect on serum osteocalcin concentration. Investigators have reported that serum osteocalcin was significantly greater in colts than fillies at weaning (Fletcher et al, 2000). However in adult horses, sex had no influence on the levels of serum osteocalcin (Lepage et al, 1997). Age has a significant inverse correlation with osteocalcin concentration in horses (Lepage et al, 1990 & 1997). This is because bone formation is higher in young horses than in adults, and gradually starts to slow down as the horse ages. In humans, serum osteocalcin is higher in men than in women and there is also an age-related decline (Kruse et al, 1986; Vanderschueren et al 1990).

Exercise has conflicting effects on the circulating osteocalcin levels. In horses, Maenpaa et al (1988) have found that the levels of serum osteocalcin decreased in foals kept in stalls compared to when they were in pasture.

Many studies in humans have also found a positive correlation between exercise and bone formation (Fujimura et al, 1997). In humans, the level of osteocalcin was reported to be 50% higher in exercised men than in non-exercised controls (Bell et al, 1988). Resistant exercise training in humans resulted in an increase in osteocalcin levels as early as the first month of exercise (Fujimura et al, 1997; Eliakim et al, 1997). Exercise may increase the osteoblastic activity, which leads to increases in the levels of osteocalcin and bone formation. It has also been reported that with additional exercise, there was no further short-term increase of osteocalcin concentration (Nishiyama et al, 1988). This may be because the osteoblastic activity increases with the increase in exercise until reaching a threshold and then will not change with additional exercise. Jackson et al (1998) on the other hand have found that treadmill exercise significantly decreased the levels of osteocalcin in the serum of two-year-old Thoroughbred horses. In a recent study, (Kawcak et al, 1998) has found that exercise did not significantly change the levels of serum osteocalcin in horses, as was found in humans (Wallace et al, 2000). In young dogs, long distance treadmill exercising did not raise the levels of serum osteocalcin (Puustjarvi et al, 1995). Conflicting results among these studies may be due to the differences in the length of each study, the type and duration of exercise program, the age of the subjects, the time of blood collection, and the osteocalcin assays used in each study.

The concentrations of osteocalcin in osteoarthritic joints have been determined in body fluids and bone samples. Gevers et al (1988) have reported that there was a significant increase in the levels of serum osteocalcin in postmenopausal women with generalized OA compared with age- and sex-matched controls. It has been shown in another study that there was an increase in osteocalcin content in iliac crest bone from patients with OA, corresponding to a shift towards higher densities in the mineralization profile (Raymaekers et al, 1992). On the other hand, Garnero et al (2001) have reported that total serum osteocalcin, measuring intact and N-mid-peptides, decreased in patients with knee OA compared with normal controls.

A positive correlation has been found between the levels of synovial fluid osteocalcin and imaging techniques including radiography (Champion et al, 1989) and bone scans (Sharif et al, 1995) in human patients with OA. The relationship between synovial fluid osteocalcin levels and the imaging techniques reflect localized increases in bone turnover. Sharif et al (1995) have suggested that synovial fluid osteocalcin levels might reflect local disease activity. This hypothesis has been supported by a study that has found a strong relationship between synovial fluid osteocalcin and keratan sulfate levels (Sharif et al, 1995). It has been shown that keratan sulfate levels are higher in synovial fluid than serum, and they mostly reflect local articular cartilage turnover (Thonar et al, 1985; Campion et al, 1991).

It has been reported that synovial fluid osteocalcin was higher than serum osteocalcin (Champion et al, 1989; Pool et al, 1991). This indicates that at least some of the synovial fluid osteocalcin is likely to be derived from local subchondral bone.

1.4.4.3.2 Biomarkers of bone resorption

Biomarkers of bone resorption can be measured in body fluids including synovial fluid, serum, and urine. The most commonly measured biomarkers of bone resorption (degradation) include hydroxyproline, glycosylated hydroxylysine, pyridinium cross-links, collagen telopeptides, and bone sialoprotein (BSP).

1.4.4.3.2.1 Hydroxyproline

Hydroxyproline is an amino acid found mainly in collagen. Thirteen to fourteen percent of the total amino acids in collagen are hydroxyproline. It is formed from the posttranslational modification of the amino acid proline by prolyl hydroxylase during collagen synthesis. Hydroxyproline has been the most widely measured marker of collagen degradation and it is concentrated mainly in urine. However, there are several problems with using this amino acid as a marker of bone resorption. First, hydroxyproline is present in all of collagens throughout the body and not specific for type I collagen of bone. Second, part of this amino acid is reabsorbed in the kidney and further degraded in the liver into products that cannot be detected by standard hydroxyproline assays. Third, the urinary level of hydroxyproline

reflects only 10-25% of collagen breakdown and the remainder comes from a breakdown of other proteins in the body. Fourth, its level is affected by diet, age, and gender and has a circadian rhythm. Fifth, about 10% of the total urinary hydroxyproline comes from newly synthesized procollagens, which is indicative of bone formation. Finally, the methods of measuring urinary hydroxyproline are difficult to perform and these include either a colorimetric assay of hydrolysed urine samples or HPLC (Kraenzin and Seibel, 1999). It has been reported that there was poor correlation between the urinary levels of hydroxyproline and bone resorption as assessed by calcium kinetics or bone histomorphometry (Delmas, 1990)

1.4.4.3.2.2 Glycosylated hydroxylysine

Glycosylated hydroxylysine (GH) is another amino acid that is unique to collagens. Unlike hydroxyproline, GH is not affected by diet and not significantly metabolized before its urinary secretion. The urinary level of GH reflects 50-100% of collagen breakdown. However, it is very difficult to measure this marker and HPLC is the method used for that (Kraenzin and Seibel, 1999). In addition, the usefulness of using GH in the evaluation of bone resorption needs validation against standard techniques such as histomorphometry.

1.4.4.3.2.3 Pyridinium cross-links

In cartilage and bone, pyridinium cross-links including pyridinoline (PYD) and deoxypyridinoline (DPD) are derived from hydroxylysine residues within the mature collagen molecule (Figure 2). An enzyme called lysyl

oxidase catalyzes the conversion of hydroxylysine residues into pyridinium cross-links, which play an important role in stabilizing collagen fibrils by covalently linking three collagen molecules together (telopeptides of two collagen molecules with one triple-helical sequence of another). In humans, the pyridinium cross-links including PYD and DPD are considered the most promising markers of bone resorption (Kraenzlin and Seibel, 1999). DPD is found in high concentrations in collagen of bone, making it a potentially specific and a more robust marker for bone resorption. On the other hand, PYD is the major cross-link in all connective tissues including the collagen of cartilage. The ratio of PYD/DPD is a useful method to distinguish between destruction of cartilage, synovium or bone. The normal ratios of PYD/DPD in cartilage, synovium, and bone are 50:1, 25:1, and 3:1, respectively. In contrast to hydroxyproline, PYD and DPD are not found in the collagens of normal skin. The pyridinoline cross-links are released when collagen breaks down and is cleared by the kidneys. Therefore, the pyridinoline cross-links are present in high concentration in urine. About 40% of total PYD and DPD in urine are present as free amino acids derivatives and 60% are present as peptide-bound fractions (Delmas et al, 1993). Although the kidneys clear the cross-links, it has been reported that renal diseases do not affect their concentration (McLaren et al, 1993). Both HPLC (Black et al, 1988) and immunoassays (Seyedin et al, 1993; Gomez et al, 1996) can be used to measure the concentration of these collagen cross-links.

In horses, the concentrations of PYD and DPD have been reported to be significantly high during the first five months of age and remain stable after that (Brama et al, 2002). Similar findings have been previously reported in humans (Eyre et al, 1984, 1988). Brama et al (2001) have showed that there was an increase in levels of PYD and DPD cross-links in non-weight bearing sites of subchondral bone that become weight-bearing sites with exercise in foals. In another study, (Kawcak et al, 2000) have found that exercise did not affect the concentration of PYD, DPD, and the PYD: DPD ratio in both the serum and synovial fluid of healthy horses. In some human studies, similar results have been found (Fujimura et al 1997; Eliakim et al, 1997). In a recent study, Brama et al (2002) have found that the concentrations of PYD and DPD in subchondral bone of foals in pasture were significantly higher than those in stall. Thus, it can be concluded that exercise has conflicting effects on bone resorption.

Hydroxypyridinium cross-links have been assessed in human patients with OA. Most studies report increased urinary concentrations of PYD and DPD in both single joint and generalized OA compared to healthy subjects (Robins et al, 1986; Black et al, 1988; Seibel et al, 1989; Thompson et al, 1992; Astbury et al, 1994). The excretion of DPD has been validated as a useful marker of bone resorption (Eastell et al, 1990). Hellio le Graverand et al (1996) have reported that there was no significant difference in PYD and DPD excretion between osteoarthritic patients and normal controls but, patients with severe or end-stage OA had higher excretion of PYD compared

to patients with early OA, while the PYD: DPD ratio remained unchanged. Another study has found that there were significantly lower levels of DPD in urine of osteoarthritic than in normal subjects (Takahashi et al, 1999). It has been reported in humans that the levels of PYD and DPD were positively correlated with the radiographic changes seen in joints with OA (Naitou et al, 2000; Takahashi et al, 1999).

Both PYD and DPD were not assessed in this study. This is because the highest concentrations of these markers are in urine samples and urine was not collected in this study. It has been reported that the levels of PYD in synovial fluid from patients with OA were about 30% of urinary levels and DPD levels were below the detection limit (Sinigaglia et al, 1995). In addition, it has been thought that there is low cross-reactivity between human and equine PYD and DPD when the ELISA is used (Kawcak et al, 2000).

1.4.4.3.2.4 Collagen telopeptides

The telopeptides refer to the short nonhelical domains at both ends of the collagen molecule (C and N-termini). In normal collagen, these telopeptides are linked to the helical portion of neighboring collagen molecule via cross-links. During collagen breakdown, C and N terminal telopeptide fragments of various sizes are released into the circulation and they are still often attached to helical portions by pyridinium cross-links (Figure 2).

1.4.4.3.2.4.1 CTXI and NTXI

CTXI and NTXI refer to the assays used to identify specific sequences of amino acids (epitopes) found in fragments of the short nonhelical telopeptides at the C- and N- terminals of type I collagen, respectively, that contain collagen cross-links (Figure 2). In normal collagen, these nonhelical telopeptides are linked via cross-links to the helical portion of neighboring collagen molecules. During collagen breakdown, the majority of C and N terminal telopeptides are relatively small fragments, which enter into the circulation. The CTXI epitope is either in a linear (nonisomerized) telopeptide form (α CTXI) or an isomerized peptide form (β CTXI). The isomerized peptide can be formed when type I collagen molecules undergo β -isomerization of the aspartic acid residue within its C-telopeptide. Changes in the level of β -isomerization might reflect metabolic status in bone diseases.

ELISAs utilizing monoclonal antibodies are available for both β CTXI and α CTXI (Bonde et al, 1994, & 1997; Fledelius et al, 1996). In urine, an ELISA was developed to measure the cross-linked part of the β CTXI using polyclonal and later monoclonal antibodies (Bonde et al, 1994; Fledelius et al, 1996). In addition, a radioimmunoassay (RIA), which is based on monoclonal antibodies, has been used to recognize α CTXI (Bonde et al, 1996). This assay shows less than 2% cross-reactivity with β CTXI (Bonde et al, 1996). More recently, a new assay for the measurement of CTXI in serum has been developed (Rosenquist et al, 1998). This assay is called CrossLaps and it is developed to quantify β CTXI in the serum and plasma. The serum

CrossLaps assay is a one step ELISA that is based on highly specific monoclonal antibodies.

A competitive inhibition ELISA has been developed to measure NTXI fragments in the urine (Hanson et al, 1990). The monoclonal antibody is specific for NTXI, which recognizes an epitope embedded in the α -2 chain of the N-telopeptide fragment and does not recognize the attached cross-links. A serum NTXI assay has been recently developed using a monoclonal antibody that is identical to the one used in the urinary assay (Woitge et al, 1999). Clinical studies have shown that these two serum assays (CTXI and NTXI) correlate well with the urinary assays and the serum NTXI assay appears to be slightly more sensitive than the serum CTXI assay in metabolic and malignant bone diseases (Woitge et al, 1999).

Several studies in humans have studied the effect of exercise on the levels of CTXI and NTXI. It has been reported in humans that exercise did not result in change for the levels of CTXI but the levels of NTXI did significantly decrease in young and adult males (Eliakim et al, 1997, Iwamoto et al, 2002). However, a recent study on young female gymnasts has found that they had significantly higher levels of CTXI compared to the controls (non-gymnasts) and concluded that gymnastics stimulates bone resorption (Jaffre et al, 2001). Although most studies have found a significant decrease in the levels of NTXI as a result to exercise, other studies have reported no difference between exercised and control groups in the levels of NTXI (Creighton et al, 2001).

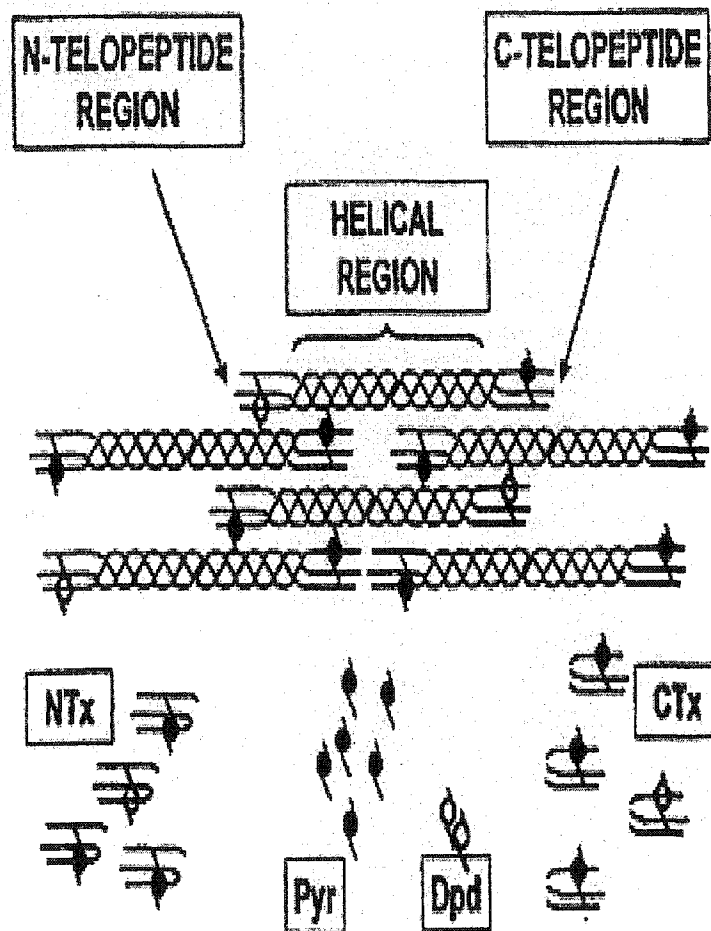


Figure 2. This figure shows several biomarkers including cross-links (pyridinoline and deoxy-pyridinoline); and cross-linked telopeptides (CTX and NTX)

The effects of osteoarthritis on the concentrations of CTXI and NTXI have been studied in humans. In a recent study, Garnero et al (2001) have found that there were significantly lower levels of both serum and urine CTXI in human patients with knee OA compared with non-arthritic controls. It has been reported that assessment of the levels of CTXI is not useful to differentiate between generalized and localized OA in humans and does not correlate with the radiographic changes seen in joints with OA (Naitou et al, 2000).

So far, these markers have not been assessed in horses. In this study, the CrossLaps CTXI assay has been used to assess serum levels of CTXI and the effects of exercise with and without osteochondral fragmentation on the levels of this marker.

1.4.4.3.2.4.2 The C-telopeptide cross-link (ICTP)

ICTP is an epitope in the carboxyterminal telopeptide region of type I collagen fragments, cross-linked via pyridinoline cross-links and liberated during the degradation of type I collagen. The ICTP assay is a radioimmunoassay based on a polyclonal antibody to measure the C-terminal cross-linked telopeptides of type I collagen (Risteli et al, 1993). Although the ICTP assay is the first assay available for assessment of bone resorption from serum samples, the results from clinical applications suggest it lacks specificity.

Age and breed have an effect on the concentration of ICTP in fluids. The concentration of ICTP is negatively correlated with age and the most

significant changes in horses are seen in the first two years of life (Lepage et al, 1998 and Price et al, 1995, 2001). The levels of ICTP have been shown to be significantly higher in draught horses than in warmblood horses (Lepage et al, 1998).

Similar to other markers, exercise has an effect on the levels of ICTP. In exercised horses, serum ICTP concentration was higher compared to non-exercised controls (Price et al, 1995). In human athletes, it has been shown that exercise caused a significant decrease in the concentration of serum ICTP (Brahm et al, 1997a), but levels rose during recovery (Brahm et al, 1997b). However in a recent study, Langberg et al (2000) have reported that exercise did not change the levels of ICTP except a short increase immediately after its cessation.

The effect of OA on the levels of ICTP has been studied in humans. It has been reported that the levels of serum ICTP did not significantly differ in patients with varying severity of OA compared to non-arthritis controls (Akimoto et al, 1997).

Serum ICTP is correlated with other markers including serum osteocalcin in both humans and horses (Lepage et al, 1998; Rong et al, 1997). In addition, the value of the osteocalcin:ICTP ratio was significantly higher in Warm Blood compared to draught horses, which might reflect a higher bone remodeling level in Warm Blooded horses due to their regular daily exercise (Lepage et al, 1998).

As mentioned above, we used the serum CrossLaps assay (CTXI) to measure bone resorption. The main reason for using this assay in this study is because it has a cross reactivity with horses based on the manufacturer's recommendations^{II}. In addition, it is a one step ELISA assay and not radioimmunoassay which makes it easy and safe to perform. The other markers of bone resorption either do not cross react, or show a weak cross-reactivity with equine epitopes.

1.4.4.3.2.5 Bone sialoprotein (BSP)

Bone sialoprotein (BSP) is a glycosylated protein that represents approximately 5-10% of the noncollagenous proteins of the bone extracellular matrix (Oldberg et al, 1985; Fisher et al, 1990). BSP is mainly produced by active osteoblasts and most of it is found in the calcified cartilage (Chen et al, 1991). The function of BSP is unknown but it is thought to play an important role in cell matrix adhesion processes and in the supramolecular organization of the extracellular matrix of mineralized tissues (Kraenzin and Seibel, 1999).

Immunoassays exist for the determination of BSP in biological fluids in humans. All of these assays are based on polyclonal antisera against bovine BSP (Sexne et al, 1995; Ohno et al, 1995; Karmatschek et al, 1997; Seibel et al, 1997).

Several studies have assessed the concentration of BSP in human osteoarthritic patients. It has been shown that there were significantly higher levels of serum BSP in osteoarthritic patients with radiographic changes

(Petersson et al, 1998a). This indicates that BSP might be a useful marker that reflects alterations in the subchondral bone turnover and remodeling following joint injury (Lohmander et al, 1996a; Conrozier et al, 1998; Wollheim, 1999). Thus, this marker has been suggested to be a potential bone marker for late stage OA (de Bri et al, 1997). Petersson et al (1998b) have shown that the concentration of serum BSP was significantly higher in osteoarthritic individuals that had bone scan abnormalities, but it did not correlate with the extent of bone scan abnormalities. With various knee injuries including OA, synovial fluid BSP increased quickly and remained high for several months after the injury (Lohmander et al, 1996a).

Although BSP is thought to be a useful marker of subchondral bone turnover and remodeling following joint injury, its level has not been assessed in this study. The reason for that is because antibodies used in the BSP assays for humans do not appear to cross-react with the equine BSP and there is no equine-specific antibody available.

1.4.4.3.3 Biomarkers of articular cartilage formation

1.4.4.3.3.1 CPII

CPII or PIICP refers to the C-terminal propeptide of type II procollagen (Figure 1). Collagen is synthesized inside the cell and secreted as a precursor procollagen molecule, which contains globular propeptide extensions at both C- and N-terminal ends. Once the procollagen is secreted, the propeptide extensions are cleaved by C- and N-proteinases and the collagen molecules are formed. One function of the propeptides is to prevent

intracellular fibril formation. Both a competitive radioimmunoassay and a recently developed ELISA have been used to assess the concentration of the CPII epitope in biological fluids. So far, the effect of exercise alone on the levels of CPII has not been studied in humans or animals.

The concentration of CPII has been evaluated in humans and horses in normal and osteoarthritic conditions. Frisbie et al (1999) measured the levels of CPII in synovial fluids and sera of horses with osteochondral fragmentation (OCF). They found that serum CPII was significantly higher in horses with OCF compared to normal controls. This suggested that there was an increase in production of type II procollagen in horses with OCF. The concentration of CPII was higher in synovial fluid from joints with OCF than in synovial fluid from healthy joints, but this increase did not reach significance. Serum CPII was higher in horses with lower grades of articular cartilage loss (grade 1 and 2) than in control horses, and was within the normal range for horses with higher grades of articular cartilage destruction (grades 3 and 4).

In humans with OA, the content of CPII in articular cartilage was found to markedly increase particularly in the mid and deep zones (Nelson et al, 1998). It has been reported that the concentration of CPII in synovial fluid of young individuals and in patients with OA was significantly higher than in adults and healthy humans, respectively (Lohmandar et al, 1996b). This reflects an increase of collagen type II synthesis in young and osteoarthritic patients. The levels of synovial fluid CPII were increased at all

stages of OA development, except in the most advanced stages where the amount of remaining articular cartilage is very low and the radiographic changes are advanced (Shinmei et al, 1993; Lohmandar et al, 1996b).

Therefore during the development of OA, the concentration of synovial fluid CPII reaches its maximum levels before the appearance of radiographic changes of OA. This increase in type II collagen synthesis might be associated with an increase in collagen II degradation, which becomes apparent during the progression of OA. It has been shown that there were significantly lower levels of CPII in synovial fluid of patients with late-stage OA compared to non-arthritic controls (Ishigurom et al, 1999). A correlation has been reported between the release of CPII from cartilage and the cartilage content (Nelson et al, 1998). It has been reported that the concentrations of synovial fluid and serum of CPII in horses were similar (Frisbie et al, 1999).

A positive correlation has been reported between CPII and other markers including CS846 (described below), bone sialoprotein (BSP), aggrecan fragments, cartilage oligomeric matrix protein (COMP), matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Lohmandar et al, 1996b).

From the above-mentioned studies, it was concluded that the levels of CPII could be utilized potentially as a simple marker of type II collagen synthesis in articular cartilage in joint diseases. In OA, CPII is thought to be

a useful marker to detect the disease in its early to moderate stages, before the appearance of radiographically-detectable changes.

1.4.4.3.3.2 Chondroitin sulfate 846 epitope (CS846)

Proteoglycans as aggrecan are a major component of the cartilage extracellular matrix. They contain a central protein core to which chondroitin sulfate and keratan sulfate chains are covalently attached. The most newly synthesized aggrecan molecules contain terminal chondroitin sulfate chains that have an epitope called chondroitin sulfate 846 (CS846) (Figure 3). The CS846 epitope is found at high levels in the fetus and its concentration declines progressively with age (Glant et al, 1986). Both competitive radioimmunoassays and, recently, an ELISA have been used to assess the concentration of CS 846 epitope in biological fluids. So far, there is no study that studies the effect of exercise on the levels of CS846 epitope in humans or in horses.

Several studies have been used to evaluate the levels of serum and synovial fluid CS846 epitope in normal and osteoarthritic joints of horses and humans. Frisbie et al (1999) have measured CS846 levels in synovial fluids and sera of horses with osteochondral fragmentation (OCF). They found significantly higher levels of CS846 in synovial fluids obtained from joints with OCF compared to fluids from non-arthritic controls. In addition, the concentration of serum CS846 was higher in horses with less severe articular cartilage loss (grade 1 and 2) and that might represent a reparative response (aggrecan synthesis). However, no significant increase was seen in

the sera of horses with severe articular cartilage loss (grades 3 and 4), which might indicate poor prognosis due to the lack of aggrecan synthesis by the chondrocytes (Frisbie et al, 1999). Similar results have been found in humans. A radioimmunoassay has been used to assess the levels of synovial fluid CS846 epitope in osteoarthritic knees (Lohmander et al, 1999). However in another study, it has been reported that there was an increase in the concentration of CS846 epitope in articular cartilage with advanced stages of OA compared to the controls (Rizkalla et al, 1992).

Correlations between CS846 and other markers have been reported in humans. Lohmander et al (1999) have found a positive correlation between the concentration of CS846 and the concentrations of CPII and COMP. In another study, a positive correlation has been reported between the concentration of CS846 epitope in articular cartilage and the histopathological changes determined by the Mankin grading system (Rizkalla et al, 1992). In general, joint fluid levels of CS846 in humans and horses are higher than in sera (Pool et al, 1994; Frisbie et al, 1999). In summary, the CS846 epitope is a marker that can be assessed in synovial fluid and serum samples using RIA or ELISA, and it is thought to reflect newly synthesized aggrecan molecules. Its level is significantly higher in OA, especially in the early stages of disease. The concentration of CS846 epitope in synovial fluid is generally higher than in serum and its concentration is positively correlated with the concentrations of other markers including CPII and COMP and with histopathological changes of articular cartilage.

1.4.4.3.4 Biomarkers of articular cartilage resorption

1.4.4.3.4.1 Direct markers

1.4.4.3.4.1.1 Sulfated glycosaminoglycans (sGAG)

sGAGs consist of variable length polyanionic carbohydrate chains with repeating disaccharide units. Six classes of glycosaminoglycans have been identified in vertebrate tissues and the three major types found in articular cartilage are chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronic acid (HA). Generally, GAG have been identified as either sulfated GAG (sGAG) such as CS and KS or nonsulfated GAGs, which includes HA.

Several methods have been used to assess the concentration of sGAGs in serum and synovial fluid. The most often used methods are the dimethylmethylene blue (DMMB) dye assay (Farndale et al, 1982; Templeton, 1988; Dey et al, 1992), and a modified alcian blue precipitation method (Little et al, 1990; Dey et al, 1992).

The effect of age on the concentration of sGAG has been assessed in several studies. In horses, Fuller et al (1996) have reported that there was no significant correlation between KS or sGAG levels and age. However, what joint was assayed significantly affected the concentration of sGAG (Fuller et al, 1996). Another study has found that sGAG contents in articular cartilage decreased with age (Brama et al, 1999). In humans with OA, Bautch et al (2000) have found that synovial fluid GAG and 3B3/GAG ratio significantly declined with aging.

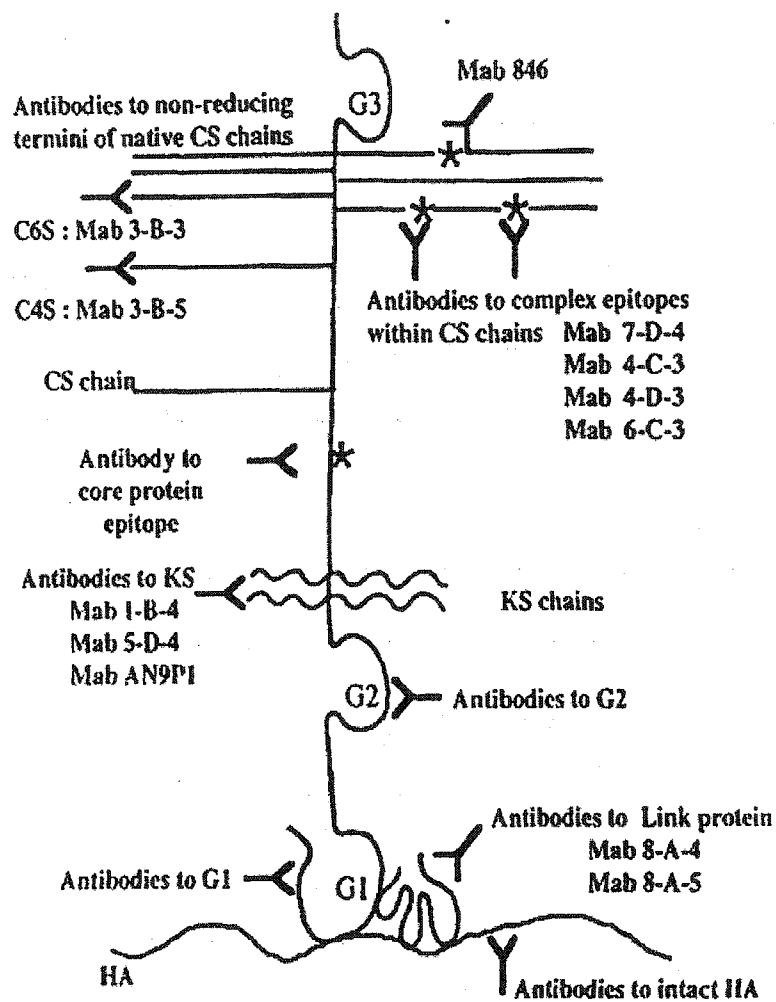


Figure 3. This figure shows the CS846 neoepitope location

The effects of exercise on sGAG concentration have been studied in humans and horses. It has been reported that the content of sGAG in cartilage from horses undergoing high intensity training was significantly higher than in cartilage from horses undergoing low intensity training (Murray et al, 2001). Brama et al (1999) have reported that sGAG content increased with increasing exercise in foals of 5 months old or less, but this increase disappeared after a further 6 months of similar exercise. It has been shown that exercise for 12 weeks did not change the concentration of sGAG in human patients with mild to moderate OA (Bautch et al, 2000).

Several studies have studied the effects of OA on the concentration of sGAG in humans and horses. Alwan et al (1991) used the DMMB to assess the levels of sGAG in synovial fluid, sera, and urine from joints with OA. The sGAG concentration in these fluids from osteoarthritic horses was generally higher than levels in fluids from non-arthritic horses and they were also higher than levels in fluids from horses with other types of joint disease (Alwan et al, 1991). The serum and urine concentration of sGAG were low compared to synovial fluid levels and there was no correlation between serum sGAG levels and those in the synovial fluid (Alwan et al, 1991). It was concluded that synovial fluid sGAG cannot be considered a specific diagnostic marker for OA because its concentration was also increased with other types of arthritis (Alwan et al, 1991). Little et al (1990), using a modified Alcian blue precipitation method, have reported that the concentration of sGAG in synovial fluid from osteoarthritic joints was

significantly higher than in the non-arthritic controls (Little et al, 1990). However, the concentration of sGAG did not correlate with the degree of articular cartilage erosion. Recently, it has been reported that there was no significant difference in the levels of synovial fluid sGAG between osteoarthritic fetlock joints and normal contralateral joints (Fuller et al, 2001). In humans, it has been found that the levels of sGAG were elevated in synovial fluid from joints with moderate to advanced OA (Ratcliffe et al, 1996). However, the overall concentrations of sGAG in normal synovial fluid were significantly higher than levels in OA synovial fluid (Ratcliffe et al, 1996).

KS has been assessed in the synovial fluid and serum of humans and animals with various joint diseases (Carroll, 1989; Alwan et al, 1990; Todhunter et al, 1993; Hazell et al, 1995; Ross et al, 1995; Bautch et al, 1997; Frisbie et al, 1999; Fuller et al, 2001). The effects of exercise and OA on the levels of KS have shown some conflicting results. Although KS levels may be useful as a marker in certain joint diseases, it has been shown that KS is not useful marker for equine OA (Todhunter et al, 1997; Frisbie et al, 1999). Thus, this marker was not assessed in this study.

Several epitopes have been identified within chondroitin sulfate. CS846 (mentioned above) and 3B3 are two examples of these epitopes located on the chondroitin sulfate. The levels of 3B3 epitope have been assessed in body fluids in humans with OA. It has been reported that the levels of synovial fluid 3B3 epitope can be used to distinguish normal from

osteoarthritic joints, but it cannot separate the different stages of OA (Ratcliffe et al, 1996). No significant effects were found with exercise for 12 weeks on the levels of 3B3, and 3B3/sGAG in human patients with mild to moderate knee OA (Bautch et al, 2000).

In conclusion, the most important sGAGs found in proteoglycans of cartilage are CS and KS. Several methods have been used to measure the levels of total sGAG in serum and synovial fluid. The level of sGAG in body fluids is considered an indicator of the degree of aggrecan turnover in articular cartilage. Since serum and synovial fluid levels of sGAG increase with various types of arthritis, this marker is considered a non-specific marker for the diagnosis of arthritis, and in particular, OA. Although some old studies have suggested that KS can be used to diagnose OA, it has been reported recently that KS is not good marker for OA. CS 3B3 epitope is a useful marker to distinguish between normal and osteoarthritic joints, but not good to differentiate among the different stages of OA.

1.4.4.3.4.1.2 COL2-3/4C_{short} and 234CEQ neoepitopes

In both normal and diseased states, collagenases cleave types I and II collagen molecules into characteristic triple helical 3/4 and 1/4 fragments. The collagenases that are able to initiate the intrahelical cleavage of triple helical collagen at neutral pH are MMP-1 (collagenase 1), MMP-8 (collagenase 2) and MMP-13 (collagenase 3) and the latter is thought to have the most activity for type II collagen (Jeffrey, 1998). MMP 14 (MTMMP-1)

can also cleave collagens at the same site as these collagenases. MMP-3 (stromelysin-1) can cleave type II fibrillar collagen but in only the nonhelical amino-telopeptide domain (Wu et al, 1991). There have been no direct assays that assess the three collagenases in equine body fluids. However, collagenase-cleaved types I and II collagens can be indirectly assessed by measuring the levels of 3/4 and 1/4 collagen fragments in body fluids. The 3/4 fragment is the large fragment that contains alpha chains with newly-created C-termini, whereas the small 1/4 fragment contains newly-created N-termini. Billinghamurst et al (1997) have developed antibodies against the newly-created C-termini (neoepitopes) found on the 3/4 α -chain fragments of type I and II collagen (COL2-3/4C_{short} neoepitope) (Figure 4). After cleavage by the collagenases, the unwinding triple helix exposes neoepitopes at the end of the newly created termini called the COL2-3/4C_{short} neoepitopes at the C-termini of the 3/4 fragment and the COL2-1/4N neoepitopes at the N-termini of the 1/4 fragments (Billinghurst et al 1997). An inhibition ELISA using polyclonal antibodies has been developed to detect the COL2-3/4C_{short} neoepitope (Billinghurst et al, 1997, 2001a). The COL2-3/4C_{short} neoepitope is considered to be a better marker of collagen cleavage than the COL2-1/4N neoepitope because the N-terminal neoepitope is usually destroyed by secondary proteolytic activity by collagenases (Billinghurst et al, 1997). COL2-3/4C_{short} neoepitope is a polyclonal antibody that detects both types I and II collagens cleaved by active collagenases. Thus, these polyclonal antibodies calculate the levels of cleaved types I and II collagens together.

In a recent study, a new antibody that specifically recognizes collagenase-cleaved type II collagen in equine cartilage, the 234CEQ neoepitope, was developed (Billinghurst et al, 2001b) (Figure 5). A neoepitope at the carboxy terminal end of the 3/4 fragment of equine type II collagen has been used to produce a polyclonal antibody. Thus, COL2-3/4C_{short} neoepitope along with the 234CEQ in body fluids might together give useful information about type I and II collagen turnover in horses (Billinghurst et al, 1997, 2001ab).

The effects of exercise on the concentration of COL2-3/4C_{short} and 234CEQ neoepitopes have not been determined. OA has an effect on the levels of both COL2-3/4C_{short} and 234CEQ neoepitopes. In an *in vitro* study, Billinghurst et al (1997) have found that there were significantly higher levels of COL2-3/4C_{short} in OA compared to adult nonarthritic cartilage. In human articular cartilage with OA, there were also higher levels of denatured type II collagen (Hollander et al, 1994). It has been reported that in both normal and osteoarthritic cartilages, there was a strong correlation between the tissue content of COL2-3/4C_{short} neoepitope and the tissue content of denatured type II collagen. These findings support the belief that collagenase activity is responsible for the cleavage and subsequent denaturation of type II collagen in human articular cartilage (Billinghurst et al, 1997). So far, the levels of COL2-3/4C_{short} in both serum and synovial fluid of osteoarthritic patients have not been measured. It has been reported that cartilage from an osteoarthritic joint of a horse had increased staining

with the 234CEQ antibody compared to nonarthritic cartilage (Billinghurst et al, 2001b). However, its levels have not yet been assessed in equine body fluids.

1.4.4.3.4.1.3 COL2-3/4m

The COL2-3/4m epitope is a hidden epitope in the intact triple helix of type II collagen and can only be exposed for antibody binding after the unwinding of the type II collagen alpha chains (Figure 4). Unwinding (denaturation) of the triple helical collagen molecule occurs after collagen cleavage that leads to further degradation by gelatinolytic proteinases (e.g. MMP-2 and MMP-9). The fragments from the denatured collagen contain the COL2-3/4m hidden epitope. An inhibition ELISA using a monoclonal antibody against the COL2-3/4m epitope is used to measure this epitope (Hollander, 1994).

1.4.4.3.4.2 Indirect markers

1.4.4.3.4.2.1 Hyaluronan (HA)

Hyaluronan is a viscoelastic nonsulfated GAG. It is found in tissues such as skin, heart valve, cartilage and the vitreous humor of the eye. Synovial membrane (particularly type B synoviocytes) has been considered the main source of the HA in synovial fluid (Yielding et al, 1957; Castor, 1957 and Roy & Ghadially, 1967; and Prehm, 1984). Synovial fluid viscosity is dependent mainly on its content of HA.

In general, the functions of HA include: 1) providing high viscosity to the synovial fluid; 2) acting as a boundary lubricant for the synovial

membrane; 3) functioning as a part of the blood-synovial barrier, 4) forming a high concentration of an adsorptive gel that prevents surface mechanical injury to the synovial membrane and articular cartilage, and 5) forming aggrecan by binding with proteoglycan molecules through link protein.

Several methods have been described for the extraction and determination of the concentration of HA in joint fluid. The mucin clot test has been used as a qualitative procedure to analyze HA-protein complexes in synovial fluid (Van Pelt, 1962; Harris & Fraser, 1971). Spectrophotometric procedures have also been used to determine the concentration of HA in a protein free solution of synovial fluid based on either estimating uronic acids using the Disches Carbazole procedure (Decker et al, 1959) or estimating hexosamine concentration using the Elson Morgan procedure (Elson and Morgan, 1933). A chromatographic procedure (Saari et al, 1989; Saari and Konttinen, 1989), and Alcian blue precipitation method (Little et al, 1990) have been also used to determine the concentration of synovial fluid HA. Recently, an inhibition ELISA has been used to assess the levels of HA in synovial fluids (Fuller et al, 2001). In general, the concentration of the HA in normal synovial fluid of horses measured by various methods is between 0.32 and 1.27 mg/ml.

Several studies have reported the concentration of HA in synovial fluid from joints of horses with various arthritides. Little et al (1990) using an Alcian blue precipitation method and Fuller et al (2001) using an inhibition ELISA quantified the concentration of HA in synovial fluid from

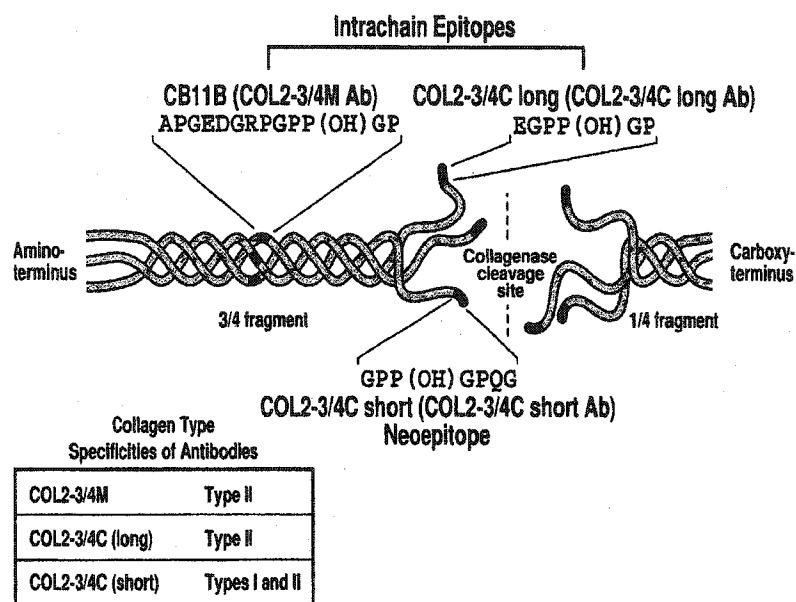


Figure 4. This figure shows the sites of the COL2-3/4C and COL2-3/4M neopeptides.

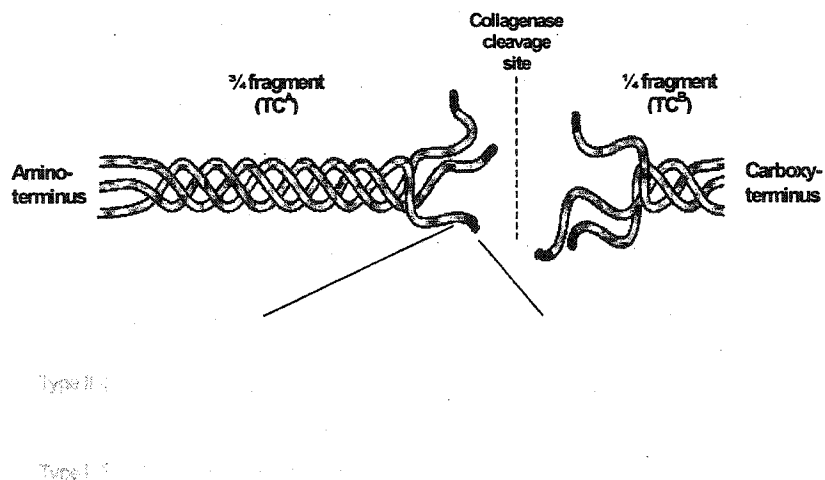


Figure 5. This figure shows the site of the 234CEQ neopeptide

osteoarthritic joints of horses. They have found that the levels of synovial fluid HA were lower than its levels in the normal control group.

Since the concentrations of HA in serum and synovial fluid change with almost all joint diseases and are not specific to OA, the levels of this marker have not been assessed in this study.

1.4.4.3.4.2.2 Cartilage oligomeric matrix protein (COMP)

COMP is one of the noncollagenous proteins found in the extracellular matrix of cartilage. The specific function of COMP is unknown but it may have a role in stabilizing the collagen network and/or in promoting collagen fibril assembly.

Exercise and OA have an effect on the concentration of COMP. With exercise, the levels of COMP have been found to decrease in dorsal sites of radial, intermediate and third carpal lateral facet in equine carpal articular cartilage compared to the palmar sites (Murray et al, 2001). It has been reported that the concentration of COMP was lower in serum from horses with osteoarthritis compared to normal controls (Skjoldebrand et al, 2001). In this study, COMP was not assessed because it is not specific for articular cartilage, but is present in other tissues including tendons, ligaments and synovium (Smith et al, 1995, 1997; Di Cesare et al, 1997).

1.4.4.3.4.2.3 Proteolytic enzymes and their inhibitors (MMPs and TIMPs)

Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes involved in both normal physiological turnover and pathological

degradation of the ECM of various tissues. In articular cartilage, these enzymes can degrade principal components of the ECM, the collagens and proteoglycans, and that may lead to diseases like osteoarthritis. MMPs are produced by a variety of cell types in the body including the chondrocytes, osteoblasts, and synoviocytes in the joint. MMPs can also be released from other cells such as neutrophils, monocytes and fibroblasts (Clegg et al, 1997). They are secreted by these cells as inactive proenzymes. In the ECM, the MMPs may be activated by various ways including plasmin and other active MMPs. Various methods have been used to measure MMP levels including ELISA, enzyme immunoassay (EIA) (Fujimoto and Iwata, 2001), zymography (Hawkes et al, 2001), activity assays using fluorogenic peptide substrates (Fields, 2001), and immunohistochemistry (Okada, 2001).

Age and disease have an effect on the level and activity of MMPs. Brama et al (1998) found that the level and activity of MMPs in synovial fluid from young horses were significantly higher than those in synovial fluids from adult horses and that may be due to declining metabolic activity in adult compared to young horses. With OA, MMP synovial fluid levels and activity were significantly higher compared to normal controls (Brama et al, 1998). This reflects an increase in ECM destruction in osteoarthritic joints compared to controls.

In an *in vitro* study, it was found that collagenolytic activities were significantly higher in cultures of both synovial membrane and articular cartilage tissues obtained from horses with moderate to severe OA

(Rechenberg et al, 2000). Gelatinolytic activities were higher in synovial membrane explant cultures than cartilage explants, whereas casinolitic activities were higher in the cartilage explant cultures (Rechenberg et al, 2000).

The proteolytic activities of MMPs can be naturally inhibited by tissue inhibitors of metalloproteinases (TIMPs) and α_2 -macroglobulin. Most MMP inhibitors are molecules that have similar sequences to the propeptides and domain substrate cleavage sites, and bind to the enzyme active sites to inactivate the MMPs. Up to date, five TIMPs have been identified: TIMP-1, -2, -3, -4, and -5. TIMPs are produced by a variety of cell types such as chondrocytes, fibroblasts, peripheral blood monocytes (Clegg et al, 1998), osteoclasts (Hill et al, 1994), and tumor cell lines (Mackay et al, 1992). In the normal situation, TIMPs bind to MMPs in a 1:1 ratio.

Similar to the MMPs, the methods used to measure TIMP levels include EIA (Fujimoto and Iwata, 2001), reverse zymography (Hawkes et al, 2001), fluorogenic peptide substrates (Fields, 2001), and immunohistochemistry (Okada, 2001). In humans with hip osteoarthritis, the level of serum TIMP-1 may help to predict progressive hip osteoarthritis (Chevalier et al, 2001). Recently, synthetic TIMPs have been used to inhibit the MMP destructive effects on the ECM (White et al, 1997). BAY 12-9566 is a synthetic MMP-inhibitor that has been used to inhibit amongst other MMPs, the destructive activity of MMP-1, MMP-8 and MMP-13 in equine articular cartilage cultures (Bilinghurst et al, 1999). Adding IL-1 to the

explant culture media stimulated the production of these MMPS. It has been found that BAY 12-9566 significantly decreased the amount of collagen cleavage products and proteoglycan released from cartilage and increased the level of proteoglycan synthesis in the explants (Billinghurst et al, 1999).

In summary, MMPs are proteolytic enzymes, produced by a variety of cell types in the body that can break down the main components of the ECM of osteochondral tissues. With arthritic diseases such as OA, the levels of these enzymes significantly increase and the ratio of MMPs to TIMPs increases. Both natural and synthetic inhibitors reduce the amount of joint destruction by inhibiting the activity of MMPs.

1.4.4.3.4.2.4 Prostaglandin E₂ (PGE₂)

One feature of inflammation is increased oxygenation of arachidonic acid which is metabolized by two enzymatic pathways, the cyclooxygenase and the 5-lipoxygenase, leading to the production of PGE₂ and leukotrienes, respectively. Two forms of cyclooxygenase have been identified: COX 1 and COX 2. COX 1 is responsible for the production of prostaglandins involved in normal physiologic functions and its level is not significantly different in inflammatory and non-inflammatory arthritis. In contrast, COX 2 expression is increased in inflammatory arthritis compared to non-inflammatory arthritis. Thus, COX 2 is the one cyclooxygenase that appears to be responsible for increased local prostaglandin production during synovitis.

It has been suggested that PGE₂ is produced by cells of the synovial membrane (May et al, 1994; Sturge et al, 1978; and Dayer et al, 1986; Wittenberg et al, 1993). The chondrocytes and bone cells also produce prostaglandins (Chin. & Lin, 1988; May et al, 1981; Raisz, 1999), but in relatively small amounts compared to synoviocytes (Wittenberg et al, 1993). Since there is no relationship between the total count of white blood cells and PGE₂ concentration, it has been suggested that PGE₂ is not produced by leukocytes (Sturge et al, 1978). Both radioimmunoassays (May et al, 1994) and enzyme immunoassays (EIA) (Gibson et al, 1996) have been used to assess the concentration of PGE₂ in synovial fluid.

The cardinal clinical sign of most joint diseases is pain and PGE₂ is considered one of the inflammatory mediators that induces pain in the joint. In addition to pain, prostaglandins (mainly the E group) cause proteoglycan depletion in the cartilage (Arnoldi et al, 1980; McDevitt & Muir, 1976; and Sweet et al, 1977). The effects of PGE₂ on joint structures also include vasodilatation, enhancement of pain perception, degradation and inhibition of proteoglycan synthesis in articular cartilage, bone demineralization, and promotion of plasminogen activator (PA) secretion (McIlwraith, 1996). Thus, PGE₂ not only causes an increase in articular cartilage destruction, but also a decrease in repair processes.

The effect of exercise on the levels of PGE₂ has been examined in several studies. Mitten et al (1995) have failed to detect PGE₂ in the plasma of exercised horses and suggested that exercise has no effect on the

concentration of plasma PGE₂. In rats however, it has been reported that acute swimming induced adrenocorticotrophic hormone (ACTH), which led to an increase in the levels of plasma PGE₂ (Watanabe et al, 1991). However, the increase in plasma levels of PGE₂ was significantly smaller with chronic exercise than with acute exercise (Watanabe et al, 1991). Exercise has become considered as one of the nonpharmacologic treatments of OA pain in humans (Creamer, 2000).

The concentration of PGE₂ has been determined in synovial fluid from osteoarthritic joints in humans and horses. May et al (1994) have assessed the concentration of PGE₂ in synovial fluid from normal and diseased joints of horses. They found that PGE₂ concentration was significantly higher in joints with OA compared to fluids from the normal joints. Gibson et al (1996), using EIA, assessed the concentration of PGE₂ in synovial fluid from normal middle carpal joints of horses and compared these with levels in fluids from pathological middle carpal joints affected by OA and chip fractures. They found that there was a significant difference in the concentration of synovial fluid PGE₂ between the control and the affected joints. In another study, Hawkins and co-workers (1993) studied the effect of inducing intra-articular synovitis in horses on the concentration of synovial fluid PGE₂. The intra-articular synovitis was induced in one antebrachial joint of each horse and the synovial fluid samples were collected several times post injection. A significant difference in the synovial fluid PGE₂ concentration between the control and the affected joints was observed 2

hours post induction of synovitis. PGE₂ levels from affected joints remained significantly higher than levels from control joints from 2 hours through 66 hours post-induction (Hawkins et al, 1993).

Kawcak et al (1997) evaluated the concentration of PGE₂ in treadmill- exercised horses with experimentally induced osteochondral fracture (OCF) in the distal aspect of the radial carpal bone of a randomly chosen intercarpal joint from each horse (N=12). Exercise was started 15 days postsurgery and the synovial fluid samples were collected pre-and several times post-surgery. One result from the study was that at all time periods, the levels of PGE₂ were significantly higher in synovial fluids from joints with OCF than in fluids from the normal controls.

In conclusion, PGE₂ in synovial fluid is produced by synoviocytes and chondrocytes, but not by white blood cells. PGE₂ increases the degradation of proteoglycans, and decreases repair processes. Levels positively correlate with the degree of lameness (pain) and are significantly higher in horses with OA compared to non-arthritic controls. We used an EIA to evaluate PGE₂ levels in our study.

1.5 Overall Summary

A synovial joint contains articular cartilage, calcified cartilage and subchondral bone. The joint is surrounded and stabilized by a joint capsule and ligaments. Changes in osteochondral tissues are often seen in normal healthy horse with exercise as an adaptive response and also in horses with

joint diseases such as OA. Osteochondral fragmentation can be employed in a useful model of equine OA.

Three techniques can be used to diagnose OA in equine joints. These include clinical examination, imaging, and the measurement of biomarker levels in body fluids. Both clinical examination and radiography are routinely used methods for detecting joint disease in its advanced stages. Therefore, new diagnostic methods are required to diagnose OA in its early stages. Nuclear scintigraphy is a highly sensitive technique but it lacks specificity. Like radiography, CT can be used to diagnose OA in its advanced but not its early stages. MRI is an excellent technique that can be used to diagnose OA in its early stages. However, it is difficult to apply this technique to horses. Therefore, more specific, sensitive and easily applicable techniques are needed to identify OA in its early stages and to monitor therapeutic response and the healing process. Measuring biomarker levels that reflect abnormalities of the turnover of bone, cartilage, and synovial tissues may be a useful method for the identification, investigation and monitoring of OA. Biomarkers are molecules of connective tissue matrices released into biological fluids during the process of tissue turnover.

In general, biomarkers of the metabolism of tissues comprising the skeletal system have been divided into those of bone, articular cartilage, and synovium metabolism. The most commonly assessed bone formation markers are BAP, PICP, PINP, and osteocalcin. The most commonly assessed biomarkers of bone resorption (degradation) include

hydroxyproline, glycosylated hydroxylysine, pyridinium cross-links, collagen telopeptides, COL2-3/4C_{short} epitope and BSP. CII and CS846 are commonly measured formation markers for articular cartilage, whereas total sGAG, KS, CS, COL2-3/4C_{short} epitope, 234CEQ neoepitope, COL2-3/4m epitope, and COMP represent the common markers used to assess articular cartilage degradation. HA, MMPs, TIMPs, and PGE₂ are markers used to evaluate the degree of synovitis in the joint.

In our study we assayed serum and synovial fluids for the levels of osteocalcin, CTXI, COL2-3/4C_{short}, CS846, CII, sGAG, 234CEQ, and PGE₂.

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2 SYNOVIAL FLUID BIOMARKERS DISTINGUISH OSTEOARTHRITIC PATHOLOGY FROM EXERCISE

2.1 The Introduction

A synovial joint contains the articulating surfaces of at least two bones covered with a thin layer of hyaline cartilage, soft tissues, and synovial fluid in the joint cavity. Hyaline cartilage is composed of an extracellular matrix containing mainly type II collagen and aggrecan. In addition to apatite crystals, bone contains an organic matrix that contains mostly type I collagen and non-collagenous proteins.

Musculoskeletal diseases of the equine athlete are a major clinical problem with consequences ranging from lost training time to catastrophic injury and loss of life (Mohammed et al, 1991; Johnson, 1993; Wilson et al, 1993). Osteoarthritis (OA) is a common musculoskeletal disorder affecting horses (Rossdale et al, 1985; Todhunter and Lust, 1990, McIlwraith, 1996). It is not only characterized by cartilage degradation, but also by changes in bone and synovium metabolism. Early diagnosis of OA would be beneficial and potentially alter the course of disease through earlier interventions.

Generally, three methods have been used to diagnose OA: musculoskeletal examination, clinical imaging techniques, and fluid biomarkers. Although clinical examination and radiographic imaging are

routine methods used to diagnose OA, they are often unable to detect this condition in its early stages. It has been reported that an approximate change in bone mineral density of 30-40% is required before bone changes can be seen in standard radiographs (Greenfield, 1986). This makes radiography an insensitive diagnostic modality. Therefore new diagnostic methods are required for diagnosing OA in its early stages.

New imaging techniques have been developed to aid in the diagnosis of OA including nuclear scintigraphy (NS), computed tomography (CT), and magnetic resonance imaging (MRI). However, these imaging techniques have some limitations when used on horses including an inability to diagnose early OA (CT), a lack of specificity (NS), and difficulty in application (MRI) (Kawcak et al, 2000). This results in the need for more specific, sensitive and applicable techniques to identify equine OA in its early stages, as well as to monitor therapeutic response and the healing process.

In normal situations there is balance between the synthesis and degradation of components required to provide articular cartilage, synovium and subchondral bone with their biomechanical and functional properties. With OA, this balance is disrupted and favors degradation to occur in the joint. In an attempt to repair the damage, it is thought that the body initially responds by increasing the synthesis of extracellular matrix components of articular cartilage (Eyre et al, 1980; Mankin and Brandt, 1992). Metabolites of the breakdown of residual molecules and the synthesis of new molecules enter the synovial fluid, blood and urine (Levick, 1992). The concentrations

of some of the metabolites can be assessed in these biological fluids and used as specific biomarkers that might be used to detect early molecular changes in OA. For each tissue in the joint, there are biomarkers for synthesis and degradation.

In addition to disease, there are other factors that affect the levels of biomarkers in biological fluids. Age and gender have effects on the levels of most of the biomarkers because tremendous changes occur in the metabolism of the immature bone and cartilage during development and maturation (Price et al, 1995a, 1997 and 2001). The health of the liver and kidney are also important because they contribute significantly to the normal clearance of most biomarkers from the circulation (Thonar et al, 1993). Load bearing and exercise markedly affect the fluid levels of biomarkers (Jackson et al, 1998; Nishiyama et al, 1988; Wallace et al, 2000; Puustjarvi et al, 1995). In general, exercise has a rapid effect on the metabolism of chondrocytes (Sah et al, 1992). Time of sampling is also an important issue because of the seasonal and diurnal variation in many marker levels (Lepage et al, 1991; Price et al, 1997, 2001).

Osteocalcin is one of the most commonly studied bone formation markers. The biological function of osteocalcin has not been precisely defined. However because of its specific interaction with hydroxyapatite, osteocalcin has been thought to be a later marker of osteoblast differentiation that is involved in regulating osteoid mineralization (Lian et al, 1982).

Conflicting reports pertaining to the effects of OA on the concentration of

osteocalcin has been reported (Gevers et al, 1988; Raymaekers et al, 1992; Garnero et al, 2001).

During bone resorption, collagen and other molecules in the organic matrix of bone are degraded by various proteases. Billingham et al (1997) have developed a polyclonal antibody, COL2-3/4C_{short} neoepitope, that detects both types I and II collagen fragments resulting from cleavage by active collagenases. It has been found that the levels of COL2-3/4C_{short} in articular cartilage from osteoarthritic joints of humans were significantly higher compared to levels in adult nonarthritic cartilage (Billinghurst et al 1997). To the best of our knowledge, the levels of COL2-3/4C_{short} in both serum and synovial fluid of osteoarthritic patients have not yet been determined. In a recent study, a new antibody that specifically recognizes collagenase-cleaved type II collagen in equine cartilage bearing the 234CEQ neoepitope, was described (Billinghurst et al, 2001b). It was reported that cartilage from an osteoarthritic joint of a horse had increased staining with the 234CEQ antibody compared to nonarthritic cartilage (Billinghurst et al, 2001b). However, levels of the 234CEQ neoepitope have not been assessed in body fluids. Thus, determining the concentration of the COL2-3/4C_{short} neoepitope along with the 234CEQ in body fluids might give useful information about the assessment of type I and II collagen turnover in health and disease (Billinghurst et al, 1997, 2001ab).

The carboxy propeptides of type II procollagen (CPII) and the chondroitin sulfate epitope 846 (CS846) have been used as biomarkers of

articular cartilage synthesis. CII has been assessed as a marker of type II collagen synthesis. The effects of osteochondral fragmentation (OCF) on the levels of CII in synovial fluid and serum have been reported in horses (Frisbie et al, 1999a). The levels of CII were higher in horses with OCF compared to those in the controls (Frisbie et al, 1999a). CS846 has been measured as a marker of aggrecan synthesis and its levels in the biological fluids obtained from joints with OA were significantly higher than those in fluids from normal controls for horses and humans (Frisbie et al, 1999a, Lohmander et al, 1999).

Several resorption markers of articular cartilage aggrecan have been evaluated in biological fluids. Sulfated glycosaminoglycan (sGAG) is a common marker that can be used as a synthetic or degradative marker of aggrecan (i.e. an aggrecan turnover marker) but since its levels have been found to be high in several joint diseases, this marker is often considered as an aggrecan degradation marker. It has been reported that the levels of sGAG were higher in the biological fluids obtained from osteoarthritic horses compared to the levels in fluids from non-affected horses (Little et al, 1990; Alwan et al, 1991).

Synoviocytes are considered rich sources of proteolytic enzymes that become active against both collagen and proteoglycan (Spiers et al, 1994). With synovitis, the levels of some inflammatory mediators increase (Spiers et al, 1994), leading to increased tissue (articular cartilage and bone) turnover. Several markers have been used to evaluate this inflammatory response in

the joint. PGE₂ is one of the most common inflammatory mediators that can be measured in the synovial fluid. It has been reported that the levels of PGE₂ were significantly higher in synovial fluid from joints of horses with OA compared to the normal levels (May et al, 1994; Gibson et al 1996).

The purpose of this study was to assess the levels of biochemical markers of bone and cartilage in synovial fluid of exercised horses with and without osteochondral fragmentation (OCF), and to determine if any of these markers could be used to differentiate the effect of exercise from those of disease on the tissue of the joint. COL2-3/4C_{short} was used as a marker of type I and II collagen degradation. CPII and 234CEQ were used as markers of type II collagen formation and degradation in articular cartilage, respectively. The levels of CS846 and sGAG were used as indicators of aggrecan synthesis and degradation in articular cartilage, respectively. The levels of cleaved type I collagen (COL1-3/4C_{short}) were calculated as the difference between the COL2-3/4C_{short} and 234CEQ levels. Osteocalcin and COL1-3/4C_{short} were used as markers of bone formation and resorption, respectively. The concentration of PGE₂ in synovial fluid was assessed as a marker of synovitis.

2.2 Materials and Methods

2.2.1 Animals

Sixteen healthy 2-year-old horses were used in this study. Eleven were females and five were males (Table 1). During a 2-week acclimatization period, all horses were dewormed and vaccinated. Prior to being admitted

into the study, body condition, lameness examination, carpal flexion test, and an effusion test were performed.

The horses were randomly assigned to either the fragmented or the control groups. The fragmented horses were subdivided into two groups: the fragmented joints (OA) and the contralateral joints (OA-normal). Therefore the horses were divided into a total of three groups: the OCF joints or OA group (n = 8 joints), the non-fragmented joints in the fragmented horses or OA-normal group (n = 8 joints), and the control joints (n = 16).

2.2.2 Osteochondral fragmentation model

In order to lessen the effects of surgery itself on the results, arthroscopic surgery was performed on both intercarpal joints of each horse. Starting 8 hours before surgery, each horse was medicated with procaine penicillin (22,000 IU/kg bw IM for 3 days) and phenylbutazone (4.4 mg/kg bw *per os* for 3 days). Each horse was sedated with xylazine, anesthetized with ketamine and diazepam, and anesthesia was maintained with halothane in 100% oxygen through a semi-closed breathing system. Each horse was positioned in dorsal recumbency and the carpal joints were flexed 90°. After sterile preparation and synovial fluid aspiration, bilateral carpal arthroscopic surgery was performed in a routine manner (McIlwraith, 1990). Two portals (each 6-10mm) were made in each joint, laterally and medially. The lateral portal was placed between the common digital extensor tendon and the extensor carpi radialis tendon and it was used for inserting the arthroscope into the joint. The medial portal was placed medial to the

extensor carpi radialis tendon and it was used for inserting the instruments. This portal allowed an 8-mm curved osteotome to be directed perpendicular to the articular cartilage surface of the radial carpal bone at the level of the medial synovial plica. After complete arthroscopic examination of the entire joint, the osteotome was used to create an osteochondral fragment in the dorsal aspect of the distal radiocarpal bone. The fragment was allowed to remain adherent to the joint capsule proximally. A motorized arthroburr was used to debride the exposed subchondral bone between the fragment and parent bone (Frisbie et al, 1997 and 1998). A 15 mm wide defect bed for the 8 mm wide fragment was created and the debris was not actively flushed from the joint. A 2-0 nylon suture was used to close the arthroscopic portals using simple interrupted or cruciate patterns. The limbs were bandaged and the horses allowed to recover. Each horse was kept in a 3.65 x 3.65 m stall. Bandages were changed every 3-5 days and maintained for two weeks. Sutures were removed 10 days postsurgery.

2.2.3 Experimental design

Figure 1 summarizes the experimental design used in this study. On day 0, before beginning the exercise program, synovial fluid samples were obtained from each horse. Exercise consisted of running on a high-speed treadmill 5 days/week; trotting for two minutes (16-19 km/h), galloping for two minutes (32 km/h), and trotting for two additional minutes (16-19 km/h). On day 21, arthroscopic surgery was performed and the horses were rested for two weeks after surgery. The exercise started again on day 35 and

continued until the end of the study (day 91). Synovial fluids were aseptically aspirated once a week from both intercarpal joints of each horse beginning on day 0 and until the end of the study (day 91). Due to a technical error, the collection of synovial fluid was missed on weeks 7 (day 49), 9 (day 63), and 11 (day 77) in all horses. From each joint, 4 ml of synovial fluid was collected and approximately 0.5 ml of this fluid was placed into a tube containing ethylenediaminetetraacetic acid (EDTA) for performing the conventional analysis of synovial fluid. The conventional analysis of synovial fluid included assessment of color, clarity, total protein, white blood cell count, differential cytology of white cells, and mucin clot formation. The remainder of the synovial fluid sample (3.5 ml) was placed into plain glass tubes, centrifuged at 3,000g for 15 minutes, and the supernatant was aliquotted in tubes and stored at -80°C for biochemical marker analysis.

2.2.4 Conventional evaluation of synovial fluid

Synovial fluid color, clarity, mucin content and blood contamination were evaluated subjectively. Color of synovial fluid was graded 1 to 5 where 1 = yellow, 2 = colorless, 3 = straw colored, 4 = orange and 5 = red (Frisbie, 1999b). Clarity was graded as 1 = clear, 2 = hazy and 3 = cloudy. Mucin content was graded 1 to 3 where 1 = good, 2 = fair, and 3 = poor (Frisbie et al, 1997). Based on blood contamination, synovial fluid samples were graded either 1=samples with no blood contamination or 2 = samples with blood contamination.

Total protein and white blood cells (WBC) concentrations were determined by using refractometry and an automated cell counter, respectively. An automated cell counter was also used to determine the differential WBC count.

2.2.5 Biomarker Assays

2.2.5.1 Bone formation marker

2.2.5.1.1 Osteocalcin

Levels of osteocalcin were measured in synovial fluid samples using a commercially available competitive immunoassay kit¹ and the manufacturer's instructions were followed to perform the assay. Briefly, 25 μ L of standards, controls, or samples were added to each well of the coated 96-well plate (already coated with human osteocalcin). The plate was then incubated for two hours at room temperature after adding 125 μ L of anti-osteocalcin antibody to each well. After washing three times with wash buffer, 150 μ L of enzyme conjugate was added to each well. The plate was incubated for one hour at room temperature and then washed three times with washing buffer. After that, 150 μ L of working substrate solution was added to each well and the plate was incubated for 40 minutes at room temperature. Finally, 50 μ L of stop solution was added to each well and the optical density was read at 405 nm using an automated reader plate. The manufacturer's instructions were followed to obtain the standard curve and calculate the levels of osteocalcin in each sample (ng/mL).

2.2.5.2 Bone resorption marker

2.2.5.2.1 COL2-3/4C_{short}

An inhibition ELISA developed by Billinghamurst et al (1997) was used to assess levels of COL2-3/4C_{short} in synovial fluid samples as a biomarker of both types I and II collagen cleavage. An Immulon 96-well plate was coated with 5ng/well of the synthetic peptide used to generate the antiserum. This peptide was conjugated to keyhole limpet hemocyanin (KLH), diluted in Tris buffer solution (TBS) at pH 7.5 and the plate was incubated overnight at 4^o C. Due to large standard deviations for recorded absorbance in outer wells, all outer wells were left uncoated and not used. After washing three times with TBS with 0.1% Tween 20 (TBS-Tween), the plate was blocked with 100 µL/well of 1% BSA in TBS (pH 7.5) for at least 30 minutes at room temperature. The plate was then washed three times with TBS-Tween and stored at 4^o C until used for sample assay. In a round bottom polypropylene 96-well plate, 50 µL/well of standards or samples and 50 µL/well of the affinity-purified COL2-3/4C_{short} antibody R2691 diluted 1:1000 in TBS containing 1% BSA and 0.1% Tween 20 (TBS-Tween-BSA) were added. In the wells serving as blanks, 50 µL/well of TBS 1%BSA and 50 µL/well of TBS-Tween-BSA were added. In the maximum binding wells, 50 µL/well of TBS 1%BSA and 50 µL/well of antibody R2691 were added. After incubation for one hour at 37^o C, 50 µL/well of the mixture was transferred to the peptide-coated ELISA plates, and incubated for 30 minutes at 4^o C.

After washing three times with TBS-Tween, 50 μ L of goat anti-rabbit IgG conjugated with alkaline phosphatase and diluted 1:20,000 in TBS-Tween-BSA was added to each well. After washing four times with TBS, 50 μ L of substrate^{II} was added to each well and the plate was incubated for 25 minutes at 37 °C. Then, 50 μ L/well of amplifier^{II} was added and the plate was incubated for 15 minutes at 37 °C. Adding 50 μ L/well of 0.3 M H₂SO₄ stopped the reaction and the optical density was read at 490 nm. A nonlinear regression equation was used to establish a sigmoidal dose response standard curve with variable slope for the determination of the concentration of COL2-3/4C_{short} in each sample (ug/mL).

The concentration of COL1-3/4C_{short} was calculated by subtracting the levels of 234CEQ in each sample from the levels of COL2-3/4C_{short} in the same sample. This allows for an approximation of the levels of cleaved type I collagen.

2.2.5.3 Articular cartilage formation markers

2.2.5.3.1 CPH

A commercially available inhibition ELISA kit^{III} was used to assess levels of the C-terminal propeptides of type II procollagen in synovial fluid samples. Based on the manufacturer's instructions, the assay was initiated by coating a Maxisorp ELISA 96-well plate with 100 μ L/well of CPH diluted 1:100 in coating buffer and incubating the plate overnight at 4 °C. After that, 50 μ L of standards or samples was added to each well of a

polypropylene 96-well plate followed by adding 50 μL /well of CPII R160 antibody diluted 1:20,000 in assay buffer and pre-incubated on a shaker for 30 minutes. After washing a coated ELISA plate three times, 80 μL /well of the mixture solution in the polypropylene plate was transferred to the ELISA plate and incubated on a shaker for two hours at room temperature. After washing the plate six times, 100 μL /well of goat anti-rabbit IgG conjugated with horseradish peroxidase and diluted 1:25,000 in 6% bovine serum albumin buffer was added and the plate was incubated for 30 minutes with shaking at room temperature. After washing six times, 100 μL of TMB (3,3',5,5'-tetramethyl benzidine) substrate /well was added and the plate was incubated with shaking for 30 minutes at room temperature. Finally, 100 μL /well of stop solution/well was added and the optical density was read at 450 nm. The manufacturer's instructions were followed to draw the standard curve and calculate the levels of CPII in each sample ($\mu\text{g/mL}$).

2.2.5.3.2 CS846

A commercially available competitive ELISA kit^{IV} was used to assess levels of the chondroitin sulfate 846 epitope in synovial fluid samples. Based on the manufacturer's instructions, this assay began by coating an ELISA 96-well plate with 100 μL /well of goat anti-mouse IgM diluted 1:500 in coating buffer and incubating overnight at 4^o C. After washing three times, the following were added to the plate: 50 μL /well of biotin-PG conjugate diluted 1:5000 in 6% BSA, 50 μL /well of PG standards or samples, and 50

μL /well of mouse anti-PG 846 antibody diluted 1:10,000 in assay buffer.

After incubating on a shaker for two hours at room temperature and washing six times, 100 μL of streptavidin horseradish peroxidase conjugate diluted 1:2500 in 6% BSA was added to each well. After incubation on a shaker for 30 minutes at room temperature and washing six times, 100 μL of substrate was added to each well. After further incubation for 30 minutes, 100 μL /well of stop solution was added and the optical density was read at 450 nm. The manufacturer's instructions were followed to create the standard curve and then calculate the levels of CS846 in each sample (ng/mL).

2.2.5.4 Articular cartilage resorption markers

2.2.5.4.1 234CEQ

An inhibition ELISA developed by Billingham et al (2001) was used to assess the levels of the 234CEQ neoepitope in synovial fluid samples. An Immulon 96-well plate was coated with 5ng/well of the synthetic peptide used to generate the 234CEQ antiserum. This peptide was diluted in Tris buffer solution (TBS) at pH 7.5 and incubated overnight at 4^o C. Due to large standard deviations in absorbance in outer wells, they were left uncoated and not used in the assay. After washing three times with TBS 0.1% Tween 20 (TBS-Tween), the plate was blocked with 100 μL /well of 1% BSA in TBS (pH 7.5) for at least 30 minutes at room temperature. After that, the plate was washed three times with TBS-Tween and stored at 4^o C. In a round bottom

polypropylene 96-well plate, 50 μ L/well of standards or samples and 50 μ L/well of the affinity purified 234CEQ antibody 5373 diluted 1:1000 in TBS containing 1% BSA and 0.1% Tween 20 (TBS-Tween-BSA) was added. In the blank wells, 50 μ L/well of TBS-Tween-BSA was added. In the maximum binding wells, 50 μ L/well of TBS 1%BSA and 50 μ L/well of antibody 5373 (234CEQ antiserum) were added. After incubating for one hour at 37 °C, 50 μ L/well of the mixture was transferred to the coated ELISA plate, and incubated for 30 minutes at 4 °C. After washing three times with TBS-Tween, 50 μ L of goat anti-rabbit IgG conjugated with alkaline phosphatase and diluted 1:20,000 in TBS-Tween-BSA was added to each well, and the plate was incubated for one hour at 37°C. After washing four times with TBS, 50 μ L of substrate^{II} was added to each well and incubated for 25 minutes at 37 °C. After that, 50 μ L/well of amplifier^{II} was added and incubated for 20 minutes at 37 °C. The reaction was stopped by adding 50 μ L/well of 0.3 M H₂SO₄ and the optical density was read at 490 nm. A nonlinear regression equation was used to establish a sigmoidal dose response standard curve with variable slope for the determination of the concentration of 234CEQ in each sample (ug/mL).

2.2.5.4.2 sGAG

A modified 1,9-dimethylmethylen blue (DMMB) dye-binding assay was used to determine GAG concentration in synovial fluid samples (Carroll, 1987, 1989). Initially, 100 μ L of each sample was placed in a tube and 100 μ L

of the digestion enzyme papain solution was added to each tube. This digestive enzyme solution was prepared by adding 32.81 gm sodium acetate and 3.72 gm disodium EDTA to double distilled water (ddH₂O) for a final volume of 1 liter and, immediately before use, 2.42 mg cysteine and 20 µL of papain suspension were added to each 1 mL of the digestion solution. After incubating the mixtures of sample and papain solution for 18 hours at 60 °C, the reaction was stopped by adding 4 µL of iodoacetamide to each tube. The stop solution can be prepared by adding 0.925 gm of iodoacetamide and ddH₂O to a final volume of 25 mL. After that, 10 µL of controls, standards, or samples were placed in duplicate in a U-bottom 96-well plate and then 190 µL of dye solution was added to each well. The optical density was read at 530 nm. To prepare one liter of dye solution, 3.04 gm of glycine, 2.37 gm sodium chloride and ddH₂O were put in a flask. After protecting the flask from the light, 16 mg of DMMB was added and stirring continued overnight. The levels of sGAG in each sample (mg/mL) were determined based on a regression equation used to draw a standard curve.

2.2.5.5 Synovitis (inflammatory) marker

2.2.5.5.1 PGE₂

Levels of PGE₂ were assessed in synovial fluid samples. This assay was performed in two major steps: PGE₂ extraction and PGE₂ evaluation.

2.2.5.5.1.1 PGE₂ extraction from synovial fluid

Reagents used for extraction of synovial fluid PGE₂ include glacial acetic acid, hexane (95% or above), ethyl acetate, ddH₂O, and 100% ethanol. Five hundred microliters of every synovial fluid sample, 500 µL of 80 % ethanol, and 10 µL of glacial acetic acid were added to a labeled eppendorf tube. The complex was mixed thoroughly, incubated at room temperature for five minutes, and centrifuged at 6000 X g for 8 minutes. An Amprep C2^V column was used to extract each synovial fluid sample. The Amprep C2 columns were inserted into the Amprep manifold and washed twice under vacuum with 1 ml of 10 % ethanol. Using a Pasteur pipette, the supernatant was withdrawn from the eppendorf tube and added, under vacuum, to the appropriately labeled column. Each column, under vacuum, was washed with 1 ml of ddH₂O, and 1 ml of hexane. At this stage, the labeled disposable culture tubes were added to the manifold for collecting column elutes. Under vacuum, each column was washed twice with 750 µL of ethyl acetate. The multi-port gas evaporation system was used to evaporate each sample. After evaporation, each tube was capped and stored at -20 °C until the ELISA was conducted.

2.2.5.5.1.2 Using ELISA to evaluate the synovial fluid PGE₂ concentration

A commercially available, competitive, high sensitivity enzyme immunoassay kit^{VI} was used to quantify the levels of PGE₂ in synovial fluid samples. The procedure is based on the manufacturer's instruction. Briefly, 150 µL and

100 μL of assay buffer were added to the non-specific binding (NSB) and the maximum binding (Bo) wells, respectively. One hundred microliters of standards or samples was added to the appropriate wells. Fifty microliters of PGE_2 HS-EIA conjugate (blue solution) was added into each well except the total activity (TA) and blank wells. After that, 50 μL /well of PGE_2 HS-EIA antibody (yellow solution) was added to all wells except the blank, TA and NSB wells. After overnight incubation at 4 $^{\circ}\text{C}$, the plate was washed three times with washing solution. Five microliters of the blue conjugate/well was added to the TA wells, and 200 μL /well of p-nitrophenyl phosphate (p-NPP) substrate was added to every well. After incubation for one hour at 37 $^{\circ}\text{C}$, 50 μL /well of stop solution were added to every well and the optical density was immediately read at 405 nm. Concentrations of PGE_2 in synovial fluid samples were determined by comparing absorbance of synovial fluid wells to absorbance of Bo, NSB, and standard wells. The manufacturer's instructions were followed to draw the standard curve and then calculate the levels of PGE_2 in each sample (pg/mL).

2.2.6 Statistical Analysis

Data normality was checked using plots of residual versus predicted values. If normality assumption was not met, the data was transformed using natural logarithm (log) and normality reverified. In cases where log transformations were required, statistics analysis was performed on the transformed data.

There were three groups in this study: OA (n = 8), OA-normal (n=8) and control group (n = 16). Having three groups is useful for determining if there was systemic effect within each group (i.e. does the fragment in one joint affect the levels of biomarkers in synovial fluid from the contralateral joint?). The OA- group included joints that had OCF in the intercarpal joints (4 in the right- and 4 in the left). The OA-normal group included the contralateral joints of the fragment horses that did not have the OCF (4 in the right- and 4 in the left intercarpal joints). The control group included horses with no OCF. The mean values between the right and left intercarpal joints in the control group were used.

A split plot with repeated measures design was used as the statistical model to evaluate the dependent variables that pertained to synovial fluid samples. These include conventional synovial fluid variables and biochemical markers of synovial fluid samples. A general linear model procedure was used to perform a multivariate analysis of variance (ANOVA) analyzing independent variables for both the main and the interaction effects on the dependent variables. The highest interaction with $P < 0.05$ was considered the most significant and was reported. When there was a significant interaction effect, a Least Square Means procedure was used to make individual comparisons.

A Pearson correlation coefficient analysis was used to study the relationship among the parameters of conventional analysis and biochemical markers in synovial fluid. Values of the correlation coefficients were

classified into four grades: slight ($r \leq 0.25$), mild ($0.25 < r \leq 0.50$), moderate ($0.50 < r \leq 0.75$), and strong ($0.75 < r \leq 1$) correlations.

2.3 Results

2.3.1 Animals

No prior lesions were noted in any joints during arthroscopic surgery except in one horse, which had partial tearing of the medial palmar intercarpal ligament in both intercarpal joints. Since no clinical signs were observed due to this lesion, this horse was not excluded from the study. For all horses, recovery from anesthesia was smooth and none required additional analgesia beyond three days of phenylbutazone administration. There were no significant differences between males and females for the synovial fluid levels of biomarkers.

2.3.2 Conventional evaluation of synovial fluid

The group, joint and time parameters had no effect on synovial fluid color and clarity. In most of the samples, the color of synovial fluid was either straw or yellow, and the clarity was clear or hazy. However, some samples had red color which was considered to be a result of blood contamination during arthrocentesis. Group ($P < 0.001$) had a significant effect on the degree of blood contamination in the synovial fluid samples (Figure 2).

This study showed that time and group had a significant effect on the WBC count ($P < 0.001$). Figures 3 shows the changes in the synovial fluid

levels of WBC count with time in the three groups. Starting on day 35, the average counts of WBC in the OA group were significantly higher than those in the other two groups. This significant difference continued until the end of the study. Starting on day 35, the average counts of WBC in the OA-normal group were higher than those in the control groups however, this increase was not statistically significant.

Group and time ($P < 0.001$) showed a significant effect in terms of the levels of total protein (TP) in the synovial fluid. The levels of synovial fluid TP were higher in the OA group compared to the other two groups (Figure 4). The significant differences between groups commenced on day 28 and continued until the end of the study.

Group ($P < 0.01$) had significant effects on the score of mucin clot in synovial fluid (Figure 5). In all the groups, mucin clot score followed a bell-shaped curve peaking on days 28 and 35. The score of mucin clot was significantly higher in the OA group compared to the nonfragmented (OA-normal and control) groups only during the first two weeks postsurgery (Figure 5).

2.3.3 Biomarkers

2.3.3.1 Formation and resorption markers of bone

2.3.3.1.1 Osteocalcin

We found that interaction between time and group had a significant effect on the concentrations of synovial fluid osteocalcin ($P < 0.001$). Figure 6

shows the changes in the levels of synovial fluid osteocalcin over time. The levels of osteocalcin increased with exercise and then decreased during the resting weeks post-surgery (days 28 and 35). One week after resuming the exercise regime (day 42), the levels of osteocalcin started to rise again and this elevation continued until the end of the study (day 91). There were significant differences between the OA group and the other two groups on day 91. The levels of osteocalcin on day 91 were slightly higher in the OA-normal group compared to the control group. However, this increase was not statistically significant. No significant differences were found in the levels of synovial fluid osteocalcin between the left and right OCF joints within the OA group.

2.3.3.1.2 COL2-3/4C_{short}

Time ($P < 0.0001$) and group ($P < 0.001$) had significant effects on the levels of synovial fluid COL2-3/4C_{short}. In addition, there were significant interactions between group and time ($P < 0.001$). Figure 7 shows the average levels of COL2-3/4C_{short} in synovial fluid samples obtained from the three groups during the study period. Five weeks after surgery (day 56), the levels of COL2-3/4C_{short} were significantly higher in the OA group compared to the non-fragmented groups. There were no significant differences between the OA-normal group and the control group or between the left and right OCF joints within the OA group.

2.3.3.1.3 COL1-3/4C_{short}

Significant effects of the group, time and their interactions ($P < 0.001$) on the levels of COL1-3/4C_{short} were found in this study. The significant differences between groups with OCF and the control group were seen five weeks postsurgery (day 56) and continued to be significant over time until the end of the study (Figure 8). No significant differences were seen between the OA-normal group and the control group or between the left and right OCF within the OA group. A strong correlation was found between the levels of synovial fluid COL2-3/4C_{short} and COL1-3/4C_{short} ($r^2 = 0.71$).

2.3.3.2 Articular cartilage biomarkers

2.3.3.2.1 CII and 234CEQ

In this study, we found that the group, time, and the interaction of these variables ($P < 0.001$) significantly affected the levels of synovial fluid CII and 234CEQ. Figures 9 and 10 show that the levels of synovial fluid CII and 234CEQ became significantly higher starting three and five weeks postsurgery, respectively and continued until the end of the study. Over the entire study, the levels of synovial fluid CII increased every week except during the resting periods (days 28 and 35), whereas the levels of 234CEQ steadily increased every week. There were no significant differences in the levels of these biomarkers between the OA-normal and the control groups or between the right and left OCF joints within the OA-group. A strong positive correlation was found between CII and 234CEQ ($r^2 = 0.67$), CII and COL2-3/4C_{short} ($r^2 = 0.77$). A very strong correlation was found between

234CEQ and COL2-3/4C_{short} ($r^2 = 0.92$). Moderate correlation was found between the levels of CII and COL1-3/4C_{short} ($r^2 = 0.55$).

2.3.3.2.2 CS846 and sGAG

Figures 11 and 12 show that time, group, group and the interactions of group and time ($P < 0.001$) significantly affected the levels of synovial fluid CS846 and sGAG. Significantly different levels of CS846 and sGAG among the various groups were observed one (day 28) and two (day 35) weeks postsurgery, respectively and continued until the end of the study. Over time, exercise alone led to increase levels of CS846 beginning with exercise and continuing until the day of surgery (day 21). Starting on day 35 until day 42, the levels of CS846 decreased and then rose again on day 56 to day 70 before decreasing until the end of the study. On the other hand, the levels of sGAG increased throughout the study. The levels of CS846 and sGAG were not significantly different between the OA-normal group and the control group or between the right and left OCF joints within the OA group. Table 2 shows the significant correlations of aggrecan metabolism markers with type II collagen metabolism markers and with the inflammatory marker PGE₂.

2.3.3.3 Inflammatory marker

Figure 13 shows the levels of synovial fluid PGE₂ within each group over time. We found that time and group ($P < 0.001$) significantly affected the levels of synovial fluid PGE₂. In addition, there were significant interactions between group and time ($P < 0.001$). Over time, the levels of

PGE₂ followed a bell-shaped curve peaking on days 28 and 35. Starting on day 28, the levels of PGE₂ were significantly higher in the OA group compared to the OA-normal and control groups. This significant increase continued until the end of the study. There were no significant differences between OA-normal and control groups and between the right and left OCF joints within the OA group.

2.4 Discussion

Synovial fluid has some advantages over other biological fluids for biomarker evaluation. Synovial fluid contains most cartilage and synovial membrane markers in their highest levels and with the longest half-lives. It is considered best for single joint disease, and levels are not affected by liver or kidney function (Alwan et al, 1991; Lohmander et al, 1992; Thonar et al, 1999). However, there are some disadvantages when evaluating markers using synovial fluid. The clearance rate has an effect on the concentration of the markers in synovial fluid (Simkin & Bassett, 1995). Inflammation increases the clearance rate from the joint fluid compartment (Wallis et al, 1987; Pejovic et al 1995; Myers et al, 1996). Thus with inflammation, there might be differences in the clearance rates of markers between the diseased and control joints. In addition, synovial fluid is relatively more difficult to obtain than serum, has a limited extractable volume, cannot be repeated within a short time frame, and requires antiseptic preparation of the joint.

Group (P < 0.001) was significantly related to the blood contamination of synovial fluid samples (Figure 2). The amount of blood contamination in

synovial fluid samples significantly increased with OA group compared to the other two groups. The red or orange color of synovial fluid indicates hemorrhage due to arthrocentesis (which is not considered a pathologic problem) or due to a pathologic disease such as acute traumatic arthritis (McIlwraith, 1980). These two situations can be distinguished by centrifuging a synovial fluid sample. If the red blood cells disappear in the pellet, this indicates that there is hemorrhage due to needle puncture. However if there is a clear sample of synovial fluid with a dark yellow or pale amber color (xanthochromic), this likely represents previous hemorrhage such as chronic traumatic arthritis. This is an indication of prolonged and low-grade hemorrhage or massive hemorrhage at some time prior to aspiration of the joint fluid (McIlwraith, 1980). In our study, there were significantly elevated levels of blood contamination of the synovial fluid on days 28, 35, 84, and 91 in OA group compared to the non-fragmented groups. However, the red blood cells disappeared in the pellets after centrifuging the contaminated synovial fluid samples. This indicates that hemorrhage was caused by needle puncture. On days 28 and 35, the possible reason for a significant increase in blood contamination in the fragmented joints was due to increased joint inflammation and increased blood supply to the joint. This is supported by the fact that the highest levels of the inflammatory marker (PGE₂) were on days 28 and 35 (Figure 13). On the other hand, repeating needle puncture was possibly the main reason for blood contamination on

days 84 and 91. This could also be due to the inflammation since PGE₂ levels were still significantly elevated in the OA groups at days 84 and 91.

In experimental OA, the color of synovial fluid samples varies from pale yellow and clear to pale yellow and opaque with some flocculent material possible (McIlwraith, 1977). In our study, we noted similar results with no flocculent material. The reason for the lack of flocculent materials might be due to the differences in the OA model used in our study.

Synovitis will often result in an increase in WBC counts in synovial fluid samples. In OA, the WBC counts vary depending on the amount of active synovitis present. In human OA, the WBC counts are typically low, whereas synovitis seems to be a more prominent feature of equine OA. In our study, we found that the numbers of WBC were significantly higher in synovial fluid from the OA group compared to that from the OA-normal and the control groups and this significant increase continued until the end of the study (Figure 3). This suggests that there was more inflammation in the joints with OCF than the contralateral and the control joints. In the model of OA that we used in this study, a motorized arthoburr was used to produce debris which was not actively flushed from the joint. This presumably contributed to the ongoing synovitis and made this model of OA an appropriate one for the production of consistent inflammation in the joint. On the other hand, the average counts of WBC in the contralateral joints were higher than those in the control joints, although this increase was not statistically significant. The explanation for this increase may be that the

horses increased the use of their contralateral joints in order to minimize the use of the painful OCF joints. This might in turn lead to increase loading of the contralateral joints and as a result mild inflammation may appear in these joints.

The levels of total protein (TP) were higher in OA group compared to those in the other two groups, and this increase was statistically significant starting on day 28 and continued until the end of the study (Figure 4). In general, the concentration of TP in synovial fluid is another indicator of synovitis. Therefore, this study showed that there was a higher level of synovitis in the intercarpal joints of exercised horses with OCF compared to those without fragments.

Mucin clot score was significantly higher in the first two weeks postsurgery in the OA group compared to the non-fragmented groups (Figure 5). It should be noted that this increase was seen while the horses were resting post surgery. When exercise started again, the score of mucin clot gradually went down over time.

In general, WBC counts, TP levels, and mucin clot score in synovial fluid are used to evaluate joint inflammation. In this study, we found that TP was the most useful indicator of inflammation compared to WBC counts and mucin clot. This is because TP levels in the synovial fluid changed very quickly post surgery and stayed significantly higher in the OA groups until the end of the study (Figure 4). The increase in synovial fluid WBC started two weeks postsurgery whereas the increases in TP and mucin clot score

started one week postsurgery (Figures 3, 4, and 5). A fluctuation in the WBC counts and mucin clot scores were seen during the study period whereas the significant increases in the levels of synovial fluid TP were relatively stable post-surgery. Although there were significant differences in WBC counts, the level of significance decreased with time (Figure 3) whereas the levels of TP were approximately the same over time. Mucin clot score increased only in the first two weeks post surgery. In addition, a higher correlation was observed between TP and PGE_2 ($r^2 = 0.26$) compared to correlations between WBC and PGE_2 ($r^2 = 0.16$) or between mucin clot score and PGE_2 ($r^2 = 0.10$). Thus, the level of synovial fluid TP appears to be more sensitive and stable indicator of joint inflammation than synovial fluid WBC counts or mucin clot.

Subchondral bone changes have been thought to play a major role in the pathogenesis of OA (Radin et al, 1995). One potential way to detect early changes in the subchondral bone is by measuring the concentrations of molecular markers of bone synthesis and resorption in body fluids. In this study, osteocalcin was measured in synovial fluid as a bone formation marker whereas $\text{COL1-3/4C}_{\text{short}}$ was assessed in synovial fluids as a bone resorption marker. To the best of our knowledge, this was the first study that assessed the levels of osteocalcin in synovial fluid of horses. We found that the concentrations of synovial fluid osteocalcin increased over time in all the groups except on days 28 and 35, which represented the only time during the study where the horses were not exercised. This indicated that exercise

alone had a positive effect on the levels of osteocalcin in synovial fluid. This suggests that exercise stimulated bone formation or the release of osteocalcin into the synovial fluid in horses. It has been thought that the sources of osteocalcin in synovial fluid include circulating osteocalcin, local production, and/or leakage into the joint from exposed subchondral bone (Campion et al, 1989; Salisbury and Sharif, 1997). Osteocalcin has a small molecular weight and therefore it might enter the joint from the circulation. Osteocalcin has affinity to hydroxyapatite which is found in the synovial fluid. It has been reported that synovial fluid osteocalcin levels were positively correlated and with the amount of hydroxyapatite crystals in synovial fluid (Campion et al, 1989; Fairney et al, 1990).

Several studies in humans and horses have found similar results. Maenpaa et al (1988) reported that the levels of serum osteocalcin decreased in foals kept in stalls compared to when they were at pasture. In humans, a positive correlation between exercise and bone formation has been reported (Fujimura et al, 1997; Eliakim et al, 1997), and the level of osteocalcin was reported to be 50% higher in exercised compared to non-exercised men (Bell et al, 1988). The exact mechanism(s) of how exercise stimulates bone synthesis is unknown, but the stress on bone due to exercise might lead the bone cells to accommodate by increasing the numbers and activities of osteoblasts, and this in turn might lead to increases in bone synthesis and osteocalcin levels.

There are studies that contradict what we found in our study.

Kawcak et al (1998) have reported that there were no significant differences in the levels of serum osteocalcin between treadmill-exercised and handwalked horses over time. However, serum samples were used in the Kawcak study, and we used synovial fluid samples in our study.

The effect of OCF on the levels of osteocalcin in synovial fluid was evaluated in this study. We found there were no significant differences in the levels of osteocalcin in synovial fluid samples among the three groups except in the last week of the study (Figure 6). This indicates that OCF has an effect on bone formation, but this effect develops slowly (10 weeks postsurgery). The mean levels of osteocalcin in the synovial fluid of the OA group were 44% higher than those from the control group. Campion et al (1989) have reported that the levels of osteocalcin in the synovial fluids of human with OA were lower than those from the normal control. Several studies have measured the levels of osteocalcin in osteoarthritic patients, but most of these studies used serum and not synovial fluid samples (Gevers et al, 1988; Raymaekers et al, 1992, Garnero et al 2001). Therefore, the results of these studies cannot be compared with our study. It can be concluded that exercise has a rapid stimulating effect on bone formation whereas osteochondral fragmentation produced detectable bone formation several weeks after the injury. In addition, this study showed that the levels of osteocalcin in OA-normal group did not change from that in the control group. This suggests that increased levels of osteocalcin in synovial fluid of one fragmented

intercarpal joint did not affect its levels in the contralateral joint of the same horse.

Ninety percent of the organic matrix in bone is type I collagen. During bone resorption, collagen and other molecules in the organic matrix of bone are degraded by various proteases. Collagenases including MMP-1 (collagenase 1), MMP-8 (collagenase 2), and MMP-13 (collagenase 3) cleave the triple helical regions of types I and II collagen molecules into characteristic 3/4-and-1/4-length fragments. MMP-13 has been expressed in equine chondrocytes (Caron et al, 1996) and is thought to be the most proteolytic enzyme for type II collagen while MMP-1 is the most active against type I collagen (Jeffrey, 1998). The concentration of COL2-3/4C_{short} neoepitope measures the levels of collagenases-cleaved types I and II collagens (Billinghurst et al, 1997, 2001a). We found in this study that exercise and OA affected the levels of synovial fluid COL2-3/4C_{short} and that these effects became significantly different starting on week 8 (day 56) after the resumption of exercise (Figure 7). This suggests that exercise and/or OCF stimulates types I and II collagen degradation. Thus, exercise and/or OCF might stimulate bone and articular cartilage resorption in equine joints. The mean levels of COL2-3/4C_{short} in the synovial fluid of the OA group were 2 fold higher than those from the control group. Billinghurst et al (1997) have found that there were significantly higher levels of COL2-3/4C_{short} in osteoarthritic compared to nonarthritic cartilage in humans. Since COL2-3/4C_{short} measures the levels of cleaved types I and II collagens together, and

234CEQ measures type II collagen (see details below), it is possible to calculate the levels of type I collagen (COL1-3/4C_{short}) by subtracting the levels of 234CEQ from the levels of COL2-3/4C_{short}. The strong correlation that we found ($r^2 = 0.71$) between COL2-3/4C_{short} and the calculated COL1-3/4C_{short} increased our confidence in using this method for determining the concentrations of COL1-3/4C_{short}. When analyzing the calculated data for COL1-3/4C_{short}, we found that there were significant differences between the OA group and the non-fragmented groups and this continued to be significant over time until the end of the study (Figure 8). This suggested that type I collagen cleavage may stimulate bone formation which occurs approximately five weeks after the detection of increases in type I collagen cleavage. This suggests that both exercise alone and exercise combined with OCF affects the synovial fluid levels of COL1-3/4C_{short}. Thus, exercise with and without OCF appear to stimulate the cleavage of type I collagen by collagenases and that leads to increased bone resorption in joints. From day 56 to the end of the study, the average significant increase of COL1-3/4C_{short} as a result of exercise and OCF was 3 fold higher than that from exercise alone. There were no significant differences between the OA-normal group and the control group. This suggests that the detectable increase in type I collagen cleavage in OCF joints has no affect on type I collagen cleavage in the healthy contralateral joints of the same horse. Therefore, when bone resorption increases in one joint as a result of OCF, the normal contralateral joint does not appear to be affected in a similar fashion.

Proteoglycans and collagens are the major components of the extracellular matrix of articular cartilage. Type II collagen is the most common collagen. CPII and 234CEQ are two markers whose levels reflect collagen synthesis and degradation, respectively (Nelson et al, 1998; Billingham et al, 2001). In this study, we found that both exercise and OA have a positive effect on the levels of CPII and 234CEQ in the synovial fluid of horses (Figures 9 and 10). These findings indicate that exercise alone stimulates type II collagen synthesis and degradation. The mean levels of CPII and 234CEQ were 70% and 1 fold, respectively higher in synovial fluid from exercised fragmented joints compared to the levels in synovial fluid from exercised-only joints. In the case of CPII, the first appearance of significant differences among groups appeared on week 6 and continued until the end of the study, whereas the significant differences among groups in the levels of synovial fluid 234CEQ were seen first on week 8 and continued until the end of the study. This suggests that detectable changes in type II collagen synthesis preceded those in its degradation by approximately two weeks. Thus, the initial response to articular cartilage injury was increased synthesis of type II collagen in the extracellular matrix. This study showed that there were no significant differences in CPII and 234CEQ levels in synovial fluid between the OA-normal group and the control group. This suggests that the increase of these markers in a fragmented joint has no effect on the levels of these markers in the normal contralateral joint of the same horse. Therefore, when articular cartilage

turnover increases in one joint as a result to OCF, the turnover of articular cartilage in the contralateral joint might not be affected. To the best of our knowledge, this is the first study that examined the effect of exercise on the concentrations of synovial fluid CPII and 234CEQ.

A study in horses has shown a significant increase in levels of serum but not synovial fluid CPII from horses with OCF compared to the control horses (Frisbie et al, 1999a). They reported that the concentrations of serum CPII and CS846 could be used to predict whether horses did or did not have OCF with a 79% success rate (Frisbie et al, 1999a). The differences in results between our study compared to what Frisbie et al have reported (1999a) may be explained by the following. In our study, the synovial fluid samples were collected weekly from horses that had similar grades of OCF whereas in the Frisbie study, the synovial fluid samples were collected only once from horses that had various grades of OCF. In our study, the synovial fluid samples were collected from only the intercarpal joints whereas in the Frisbie study, they were collected from both intercarpal and radiocarpal joints. Furthermore, radioimmunoassay was used to measure the levels of CPII in the Frisbie study whereas ELISA was used in our study. The location of OCF in our study was in the distal radiocarpal bone, which is the most common site in the carpal joint for natural OCF and the fragment involved both the articular cartilage and the subchondral bone. In the Frisbie study, the OCF lesions were in various locations within the intercarpal and radiocarpal joints. In the Frisbie study, the horses had natural lesions of

OCF whereas experimental OCF was induced in the horses used in our study. All these reasons might be responsible for the differences in results between the two studies.

Billingham et al (2001b) have reported that cartilage from an osteoarthritic joint of a horse had increased staining with the 234CEQ antibody compared to staining of nonarthritic cartilage.

Proteoglycans, as aggrecan molecules, are considered the other major structural component of articular cartilage. CS846 and sGAG are two markers that routinely reflect proteoglycan synthesis and turnover, respectively (Alwan et al, 1991; Rizkalla et al, 1992; Frisbie et al, 1999). In this study, we found that the levels of synovial fluid CS846 and sGAG significantly increased with both exercise and OA.

With exercise alone, the levels of CS846 increased from one week to the next during the first month of the study, and then they decreased until day 70 before gradually increasing until the end of the study (Figure 11). This suggests that aggrecan synthesis increased with exercise. To the best of our knowledge, this is the first study that determined the effects of exercise on the concentrations of synovial fluid CS846 in horses.

The concentration of synovial fluid sGAG increased with exercise alone during this study. This increase in synovial fluid sGAG levels was higher in every week compared to the previous week during the study period. This suggests that exercise alone may stimulate increased proteoglycan turnover in the articular cartilage of healthy horses. In a recent study,

Murray et al (2001) have reported that the content of sGAG in cartilage from horses undergoing high intensity training was significantly higher than in cartilage from horses undergoing low intensity training. Brama et al (1999) have reported that sGAG content increased with increasing exercise in five-month-old foals and younger, but this increase disappeared after an additional 6 months of similar exercise.

Of the biomarkers assessed in this study, CS846 and sGAG may provide the earliest and most useful information concerning the presence and development of OA. The significant difference in the levels of CS846 and sGAG between the OA group and the other two groups started one and two weeks post- surgery, respectively (Fig 11 and 12). The mean levels of CS846 and sGAG were 1 fold and 40%, respectively higher in synovial fluid from exercised fragmented joints compared to the levels in synovial fluid from exercised-only joints.

Studies in humans and horses with joint diseases have shown an increase in body fluid levels of CS846 epitope with disease (Rizkalla et al, 1992; Mansson et al, 1995; Bassleer et al, 1996; Frisbie et al, 1999; Ishigurom et al, 1999; Lohmander et al, 1999). Frisbie et al (1999) found significantly higher levels of CS846 in synovial fluid and serum from horses with OCF compared to the levels in fluid from unaffected horses.

The concentrations of sGAG in OA fluids have been evaluated in humans and horses. Alwan et al (1991) used the DMMB assay to assess the levels of sGAG in synovial fluid, sera, and urine from joints with OA. They

found that the concentrations of sGAG in these fluids from osteoarthritic horses were generally higher than levels in fluids from non-arthritic horses and they were also higher than levels in fluids from horses with other types of joint disease (Alwan et al, 1991). The synovial fluid levels of sGAG were significantly higher for OA joints than normal, septic, and joints with osteochondrosis. It was concluded that synovial fluid sGAG cannot be considered a specific diagnostic marker for OA because its concentration was also increased although not significantly with other types of arthritis (Alwan et al, 1991). Little et al (1990), using a modified alcian blue precipitation method, also found that the concentration of sGAG in synovial fluid from osteoarthritic joints was significantly higher than non-arthritic controls. However, the concentration of sGAG did not correlate with the degree of articular cartilage erosion. Although sGAG has been used as an aggrecan degradation biomarker, it should be noted that this is not quite correct because a portion of the total sGAG molecules in body fluid are considered newly synthesized sGAG.

A moderate positive correlation between sGAG and PGE_2 ($r^2 = 0.36$) suggests that PGE_2 may play a role in the depletion of proteoglycans from articular cartilage. Since the correlation between these two markers was not very high, there are other factor(s) (e.g. interleukin-1 and tumor necrosis factor) along with PGE_2 that stimulate proteoglycan depletion in early OA. A moderate correlation between CS846 and PGE_2 suggests that

inflammation not only is associated with aggrecan resorption, but also is related to synthesis of articular cartilage aggrecan.

A strong positive correlation between CS846 and sGAG in synovial fluid ($r^2=0.46$) was found in this study. Therefore it can be concluded that during early OA, there is an increase in proteoglycan turnover. Positive correlations between sGAG and 234CEQ ($r^2=0.49$), between CS846 and CII ($r^2=0.50$), between CS846 and 234CEQ ($r^2=0.37$), and between sGAG and CII ($r^2=0.64$) suggest that collagen turnover is associated with the proteoglycan turnover. However, it appears that, based on the synovial fluid levels of specific biomarkers, proteoglycan turnover preceded the collagen turnover. Therefore, this study supported the hypothesis suggesting that proteoglycan turnover is the earlier sign observed in the osteoarthritic articular cartilage and it precedes the turnover of type II collagen (Billinghurst et al, 2000). Most studies that have measured biomarkers levels for the purpose of diagnosing early OA have targeted increases in levels of markers of degradative biomarkers from articular cartilage. In this study, we found that aggrecan and collagen synthesis preceded the synovial fluid levels of biomarkers of their degradation in early OA. This suggests that increased aggrecan synthesis is the earlier sign that can be detected in osteoarthritis.

This study showed that in early OA, the changes in aggrecan metabolism biomarkers (CS846 and sGAG) and type II collagen synthesis (CII) preceded those in bone metabolism biomarkers (osteocalcin and

COL1-3/4C_{short}) by at least a month. This suggests that articular cartilage turnover preceded bone turnover in equine joints with early OA.

Prostaglandins (primarily E group) are inflammatory mediators produced by synoviocytes in inflamed joints and have been shown to cause proteoglycan depletion in the cartilage matrix (Arnoldi et al, 1980; Lippello et al, 1978; McDevitt & Muir, 1976; Robinson et al, 1976, Sweet et al, 1977). In this study, we found that exercise and OA affected the levels of synovial fluid PGE₂. These results differ from what Mitten et al (1995) reported. They could not detect a significant increase in the levels of plasma PGE₂ as a result to exercise in horses and therefore, they concluded that exercise had no effect on the concentration of plasma PGE₂. In fact, the levels of PGE₂ in blood are very low compared to synovial fluid. Therefore, the levels of PGE₂ in blood may be below the detection limit of the assay. Furthermore, the magnitude and duration of exercise and the assay used to measure the levels of plasma PGE₂ in that study are different from what we used in our study. In another study, Watanabe et al (1991) have found that plasma levels of PGE₂ in rats were significantly lower with chronic exercise compared to acute exercise.

In this study, we found that the concentration of PGE₂ was significantly higher in synovial fluid samples obtained from joints with OCF compared to the normal joints. Kawcak et al (1997) have reported similar results in horses using the same OA model. Other studies with horses have also reported that the levels of synovial fluid PGE₂ were significantly higher

in equine joints with OA compared to non-arthritic controls (May et al, 1994; Gibson et al, 1996). Increasing levels of PGE₂ in osteoarthritic joints is a useful sign indicating that there is increasing soft tissue inflammation (synovitis) and likely pain in the joint. Therefore, exercise alone and in conjunction with OA causes inflammation in the joint. Increased levels of PGE₂ in synovial fluid of equine joint with OCF did not significantly influence levels in the normal contralateral joint for the same horse.

In summary, this study measured the levels of molecular markers of bone and articular cartilage metabolism in synovial fluid samples obtained from exercised horses with or without experimentally induced OA. Based on the evaluation of synovial fluid levels of these biomarkers, both exercise and OA stimulated cartilage and bone metabolism. Our study showed that significant changes in the levels of synovial fluid biomarkers of articular cartilage metabolism preceded those of bone metabolism in equine joints with early OA by at least a month. Within the group of articular cartilage biomarkers evaluated in this study, those indicating aggrecan turnover increased before those indicating type II collagen turnover. Articular cartilage synthesis preceded its degradation in equine joints with early OA. In addition to articular cartilage degradation, increased levels of PGE₂ were related to increases in articular cartilage synthesis. Inflammation due to exercise and/or OCF may be the key factor influencing the synovial fluid levels of these biomarkers. The significant increases in inflammation due to OCF, as suggested by the significant increases in WBC counts, total protein

and PGE₂ levels in the synovial fluids of the OA group, may account for the significant increases noted in the OA group for most biomarkers evaluated in this study. The positive correlations that we found between these biomarkers and PGE₂ levels in synovial fluid support this finding.

2.5 Tables and Figures

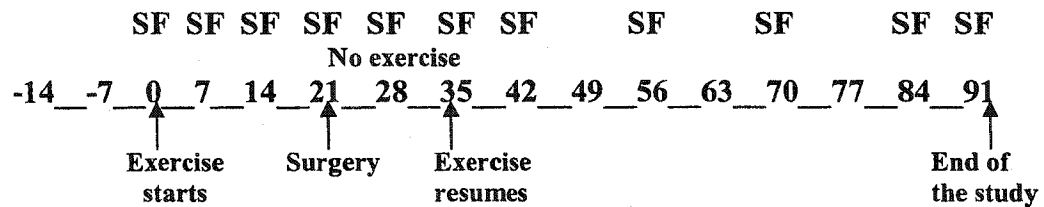


Figure 1. The timeline of this study is summarized in this figure. The study starts two weeks before the beginning of the exercise (-14) and finished on day 91. On day 21, surgery was performed on each horse. After that, the horses were rested for two weeks. Then, the exercise resumed and continued until the end of the study. Synovial fluids (SF) were aseptically collected once a week from right and left mid carpal joints of each horse beginning on day 0 and until the end of the study (day 91) except on weeks 7 (day 49), 9 (day 63), and 11 (day 77).

Table 1. The gender of the horses and the side of OCF in each fragmented joint. Both gender and side had no significant effect on the levels of biomarkers.

Group/Gender	Male	Female
Left OCF	2	2
Right OCF	1	3
Control	2	6

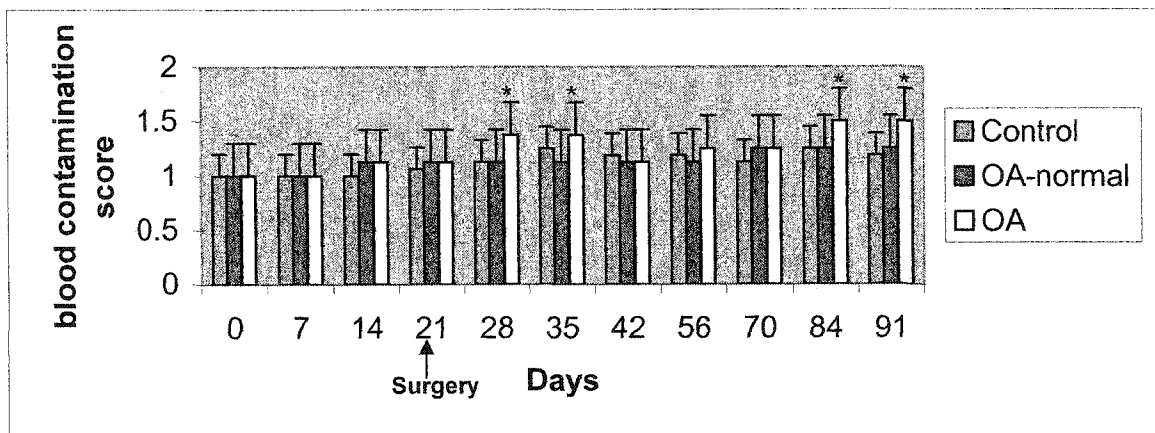


Figure 2. The mean ($\pm$ SEM) blood contamination score. On days 28, 35, 84 and 91, the average blood contamination score was significantly higher in the OA group compared to the non-fragmented groups. There was no significant difference between the OA-normal and the control groups. The * indicates significantly different blood contamination scores for OA group versus non-fragmented groups ($P < 0.05$).

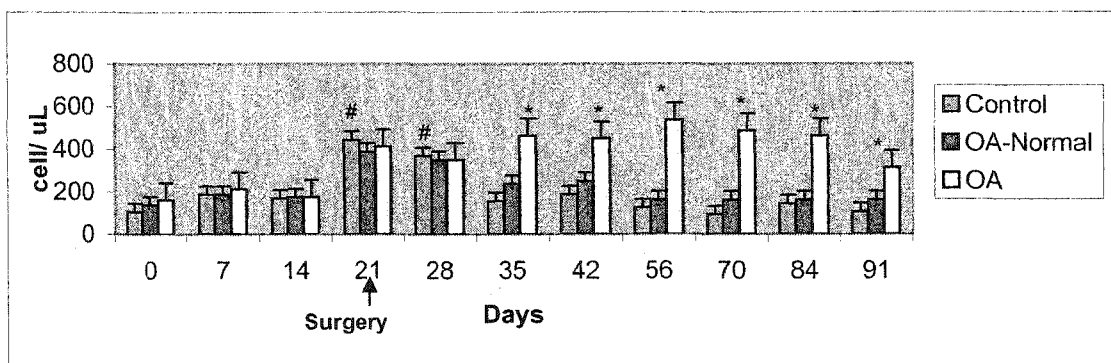


Figure 3. The mean (± SEM) counts of WBC in SF. On day 35, the average counts of WBC in the OA group were significantly higher than those in the other two groups. This significant difference continued until the end of the study. Starting on day 35, the average counts of WBC in OA-normal were slightly higher than that in the control group, however, this increase was not statistically significant. The * indicates the significantly elevated WBC counts in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher numbers of WBC compared to day 0 in the control group ($P < 0.05$).

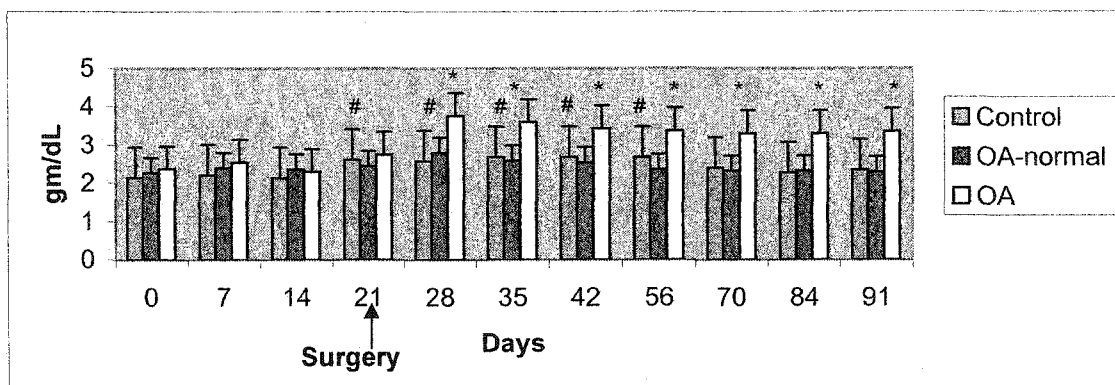


Figure 4. The mean (± SEM) concentrations of total protein in SF. Starting on day 28, The average levels of total protein were significantly higher in the OA group compared to the other two groups. The significant increase continued until the end of the study. Starting on day 28, the levels of total protein in SF from OA-normal were slightly higher than that from the control groups, however, this increase was not statistically significant. The * indicates the significantly elevated total protein levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of TP compared to day 0 in the control group ($P < 0.05$).

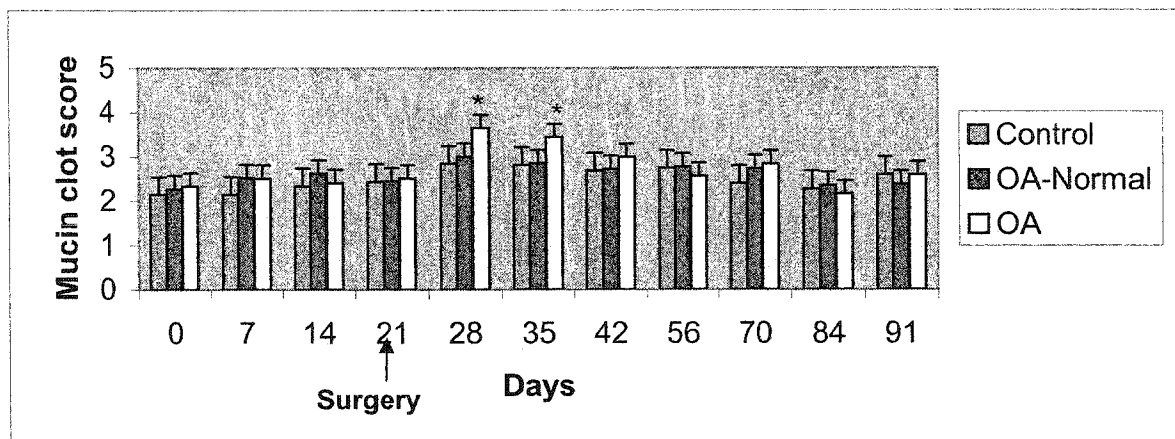


Figure 5. The mean (± SEM) scores of mucin clot in SF. On days 28 and 35, the score of mucin clot was significantly higher in the OA group compared to the other two groups. There is no significant difference between the OA-normal and the control groups. The * sign indicates the significantly elevated mucin clot scores for the OA group versus the other two groups ($P < 0.05$).

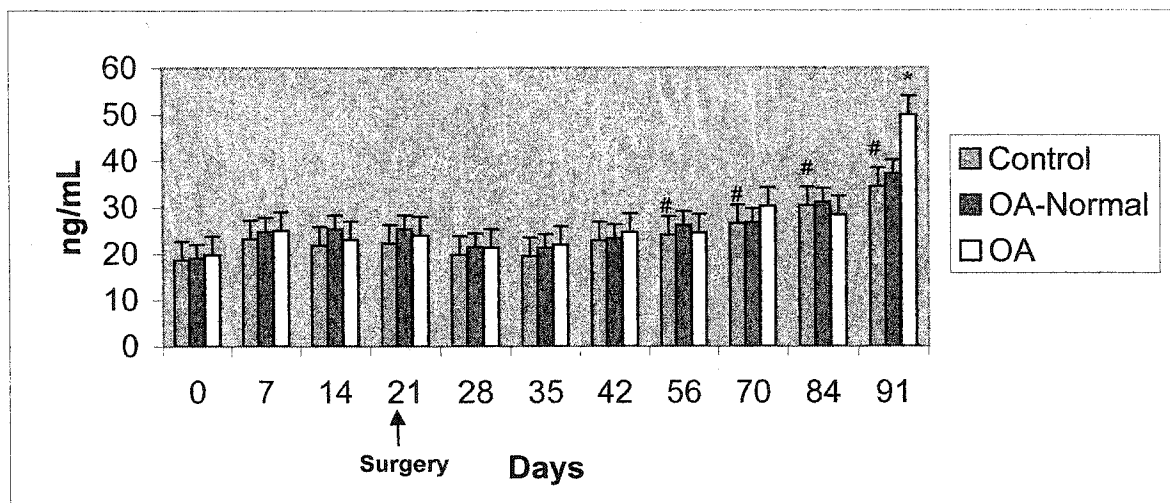


Figure 6. The mean (± SEM) concentration of osteocalcin in SF. The levels of osteocalcin increased with exercise and then decreased during the resting weeks (weeks 4 and 5). One week after starting the exercise again (day 42), the levels of osteocalcin started to rise up and continued increasing until the end of the study (day 91). On day 91, the levels of osteocalcin was significantly higher in the OA group compared to the other two groups. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared with the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of osteocalcin compared to day 0 in the control group ($P < 0.05$).

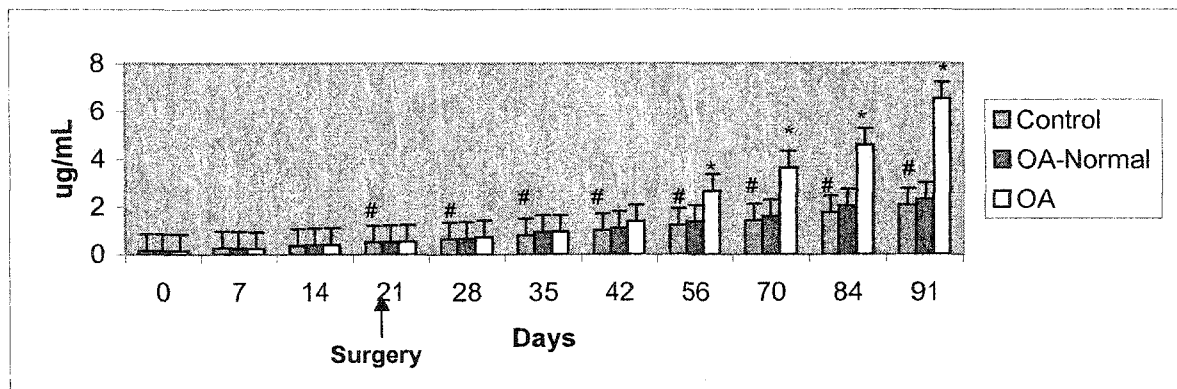


Figure 7. The mean ($\pm$ SEM) concentrations of COL2-3/4Cshort in SF. Starting on day 56, the levels of COL2-3/4Cshort were significantly higher in the OA group compared to the other two groups. The significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of COL2-3/4Cshort compared to day 0 in the control group ($P < 0.05$).

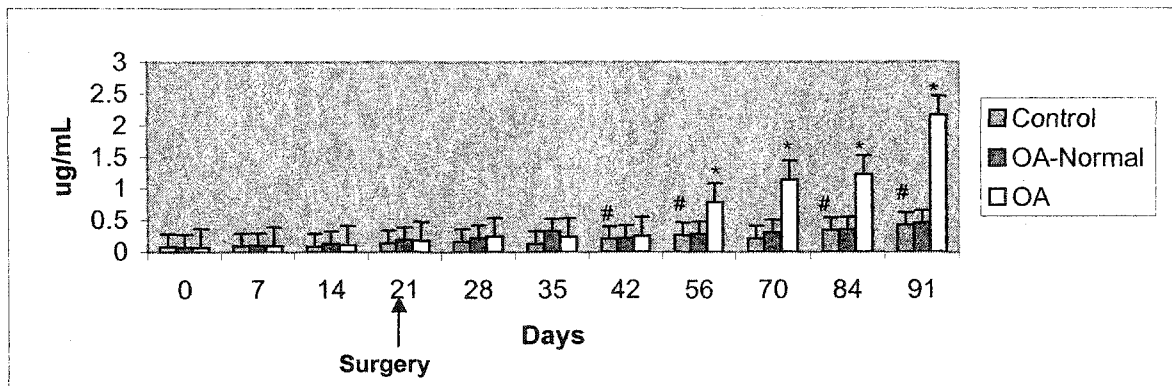


Figure 8. The mean ($\pm$ SEM) concentrations of COL1-3/4Cshort in SF. Starting on day 56, the levels of COL1-3/4Cshort were significantly higher in the OA group compared to the other two groups. The significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of COL1-3/4Cshort compared to day 0 in the control group ($P < 0.05$).

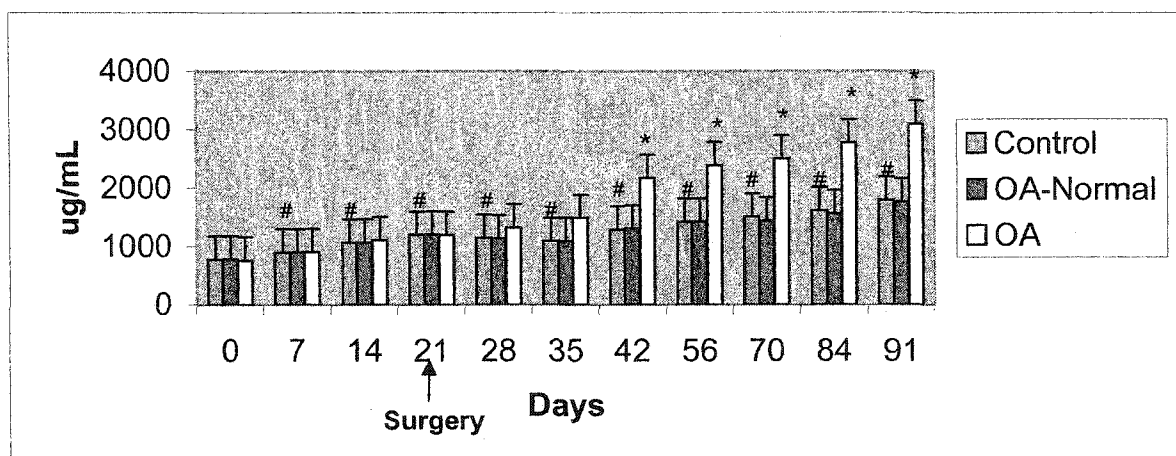


Figure 9. The mean (± SEM) concentrations of CPII in SF. Starting on day 42, the average levels of CPII were significantly higher in the OA group compared to the other two groups. This significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels for the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of CPII compared to day 0 in the control group ($P < 0.05$).

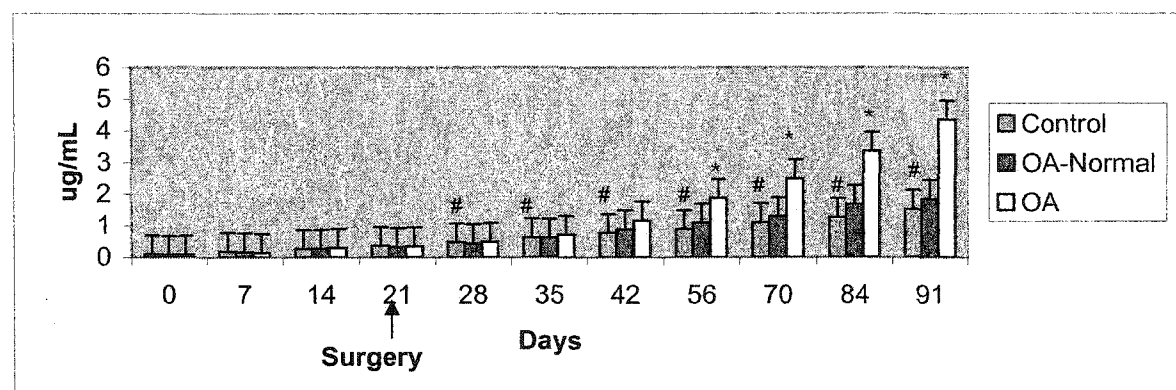


Figure 10. The mean (± SEM) concentrations of 234CEQ in SF. Starting on day 56, the levels of 234CEQ were significantly higher in the OA group compared to the other two groups. This significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels for the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of 234CEQ compared to day 0 in the control group ($P < 0.05$).

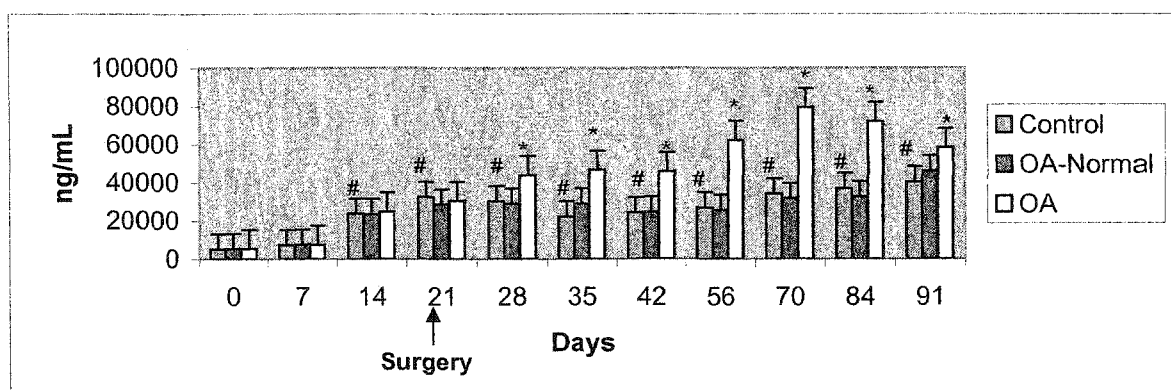


Figure 11. The mean ($\pm$ SEM) concentrations of CS846 in SF. Starting on day 28, the levels of sGAG were significantly higher in the OA group compared to the other two groups. This significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of CS846 compared to day 0 in the control group ($P < 0.05$).

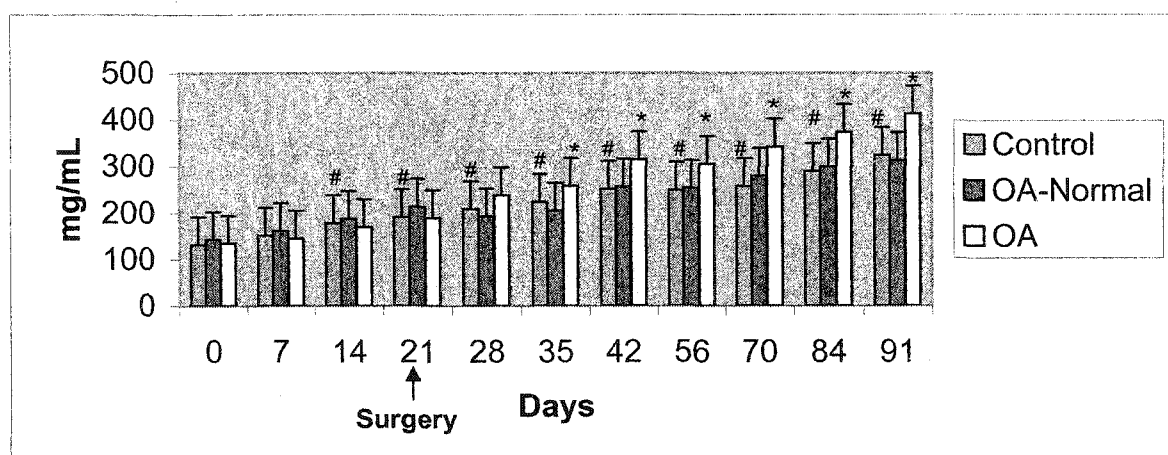


Figure 12. The mean ($\pm$ SEM) concentrations of sGAG in SF. Starting on day 35, the levels of sGAG were significantly higher in the OA group compared to the other two groups. This significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of sGAG compared to day 0 in the control group ($P < 0.05$).

Table 2. This table shows values of R^2 between the biomarkers CS846 and sGAG with the other markers in the synovial fluid.

	CS846	sGAG	CPII	234CEQ	PGE2
CS846	1	0.46	0.5	0.37	0.36
sGAG	0.46	0.46	0.64	0.49	0.36

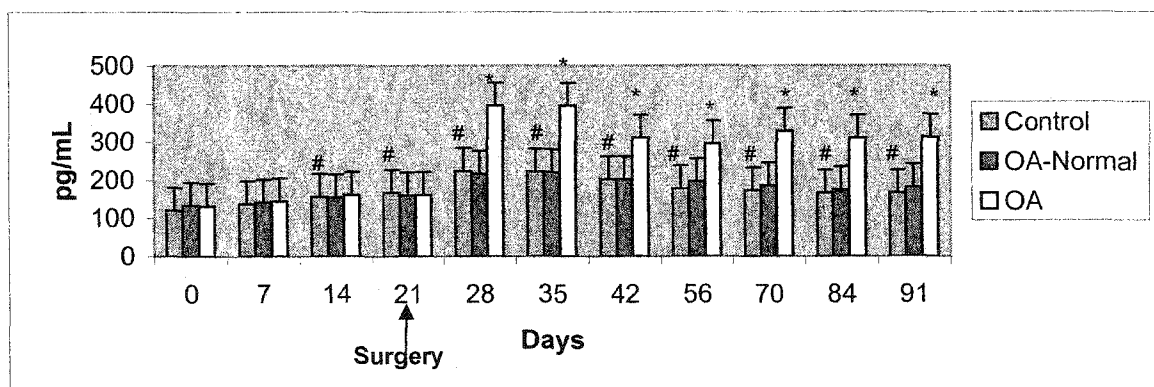


Figure 13. The mean ($\pm$ SEM) concentrations of PGE₂ in SF. Starting on day 28, the average levels of PGE₂ were significantly higher in OA group compared to the other two groups. The significant increase continued until the end of the study. There was no significant difference between the OA-normal and the control groups. The * indicates the significantly elevated levels in the OA group compared to the other two groups ($P < 0.05$). The # indicates the days that significantly have higher levels of PGE₂ compared to day 0 in the control group ($P < 0.05$).

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I. Novocalcin, Metra Biosystems, Inc, CA, USA

II. ELISA Amplification System, GIBCO BRL, Grand Island, NY, USA

- III. Collagen II pro-peptide ELISA, HDM diagnostic & imaging inc, Toronto, Canada
- IV. CS846 ELISA assay, HDM diagnostic & imaging inc, Toronto, Canada
- V. Amprep C2 column, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA
- VI. High sensitivity PGE₂ enzyme immunoassay kit, Assay Designs Inc, Ann Arbor, MI, USA.

3 SERUM BIOMARKERS DISTINGUISH OSTEOARTHRITIC PATHOLOGY FROM EXERCISE

3.1 The Introduction

A synovial joint contains the articulating surfaces of at least two bones covered with a thin layer of hyaline cartilage, soft tissues, and synovial fluid in the joint cavity. Hyaline cartilage is composed of an extracellular matrix containing mainly type II collagen and aggrecan. In addition to the apatite crystals, bone contains an organic matrix that contains mostly type I collagen and non-collagenous proteins.

Musculoskeletal diseases of the equine athlete are a major clinical problem with consequences ranging from lost training time to catastrophic injury and loss of life (Mohammed et al, 1991; Johnson, 1993; Wilson et al, 1993). Osteoarthritis (OA) is a common musculoskeletal disorder affecting horses (Rossdale et al, 1985; Todhunter and Lust, 1990; McIlwraith, 1996). It is not only characterized by cartilage degradation, but also by changes in bone and synovium metabolism. Earlier diagnosis of OA would be beneficial and potentially alter the course of disease through earlier interventions.

Generally, three methods have been used to diagnose OA: musculoskeletal examination, clinical imaging techniques, and fluid biomarkers. Although clinical examination and radiographic imaging are

routine methods used to diagnose OA, they are often unable to detect this condition in its early stages. It has been reported that an approximate change in bone mineral density of 30-40% is required before bone changes can be seen in standard radiographs (Greenfield, 1986). This makes radiography an insensitive diagnostic modality. Therefore new diagnostic methods are required for diagnosing OA in its early stages.

New imaging techniques have been developed to aid in the diagnosis of OA including nuclear scintigraphy (NS), computed tomography (CT), and magnetic resonance imaging (MRI). However, these imaging techniques have some limitations when used on horses including an inability to diagnose early OA (CT), a lack of specificity (NS), and difficulty in application (MRI) (Kawcak et al, 2000). This results in the need for more specific, sensitive and applicable techniques to identify equine OA in its early stages, as well as to monitor therapeutic response and the healing process.

In normal situations there is balance between the synthesis and degradation of components required to provide articular cartilage, synovium and subchondral bone with their biomechanical and functional properties. With OA, this balance is disrupted and favors degradation to occur in the joint. In an attempt to repair the damage, it is thought that the body initially responds by increasing the synthesis of extracellular matrix components of articular cartilage (Eyre et al, 1980; Mankin and Brandt, 1992). Metabolites of the breakdown of residual molecules and the synthesis of new molecules enter the synovial fluid, blood and urine (Levick, 1992). The concentrations

of some of the metabolites can be assessed in these biological fluids and used as specific markers that might be used to detect early molecular changes in OA. For each tissue in the joint, there are biomarkers for synthesis and degradation.

In addition to disease, there are other factors that affect the levels of biomarkers in biological fluids. Age and gender have effects on the levels of most of the biomarkers because tremendous changes occur in the metabolism of the immature bone and cartilage during development and maturation (Price et al, 1995a, 1997 and 2001). The health of the liver and kidney are also important because they contribute significantly to the normal clearance of most biomarkers from the circulation (Thonar et al, 1993). Load bearing and exercise markedly affect the fluid levels of biomarkers (Jackson et al, 1998; Nishiyama et al, 1988; Wallace et al, 2000; Puustjarvi et al, 1995). In general, exercise has a rapid effect on the metabolism of chondrocytes (Sah et al, 1992). Time of sampling is also an important issue because of the seasonal and diurnal variation in many marker levels (Lepage et al, 1991; Price et al, 1997, 2001).

Osteocalcin is one of the most commonly studied bone formation markers. The biological function of osteocalcin has not been precisely defined. However because of its specific interaction with hydroxyapatite, osteocalcin has been thought to be a later marker of osteoblast differentiation that is involved in regulating osteoid mineralization (Lian et al, 1982). Conflicting reports pertaining to the effects of OA on the concentration of

osteocalcin has been reported (Gevers et al, 1988; Raymaekers et al, 1992; Garnero et al, 2001).

Several markers of bone resorption have been developed and the most recent assays are the Serum CrossLaps (CTXI) (Rosenquist et al, 1998) which detects a cross-linked telopeptide fragment of type I collagen and COL2-3/4C_{short} neoepitope (Billinghurst et al, 1997) which detects both types I and II collagen fragments resulting from cleavage by active collagenases. Garnero et al (2001) have found that there were significantly lower levels of both serum and urine CTXI in human patients with knee OA compared with non-arthritic controls. It has been found that the levels of COL2-3/4C_{short} in articular cartilage from osteoarthritic joints of humans were significantly higher compared to levels in adult nonarthritic cartilage (Billinghurst et al 1997). So far, these resorption markers of bone have not been assessed in horses.

In a recent study, a new antibody that specifically recognizes collagenase- cleaved type II collagen in equine cartilage, the 234CEQ neoepitope, was described (Billinghurst et al, 2001b). It was reported that cartilage from an osteoarthritic joint of a horse had increased staining with the 234CEQ antibody compared to nonarthritic cartilage (Billinghurst et al, 2001b). However, its levels have not been assessed in body fluids. Thus, the COL2-3/4C_{short} neoepitope along with the 234CEQ in body fluids might give great information about the assessment of type I and II collagen turnover (Billinghurst et al, 1997, 2001ab).

The carboxy propeptides of type II procollagen (CPII) and the chondroitin sulfate epitope 846 (CS846) have been used as biomarkers of articular cartilage synthesis. CPII has been assessed as a marker of type II collagen synthesis. The effects of osteochondral fragmentation (OCF) on the levels of CPII in synovial fluid and serum have been reported in horses (Frisbie et al, 1999a). The levels of CPII were higher in horses with OCF compared to those in the controls (Frisbie et al, 1999a). CS846 has been measured as a marker of aggrecan synthesis and its levels in the biological fluids obtained from joints with OA were significantly higher than those in fluids from normal controls for horses and humans (Frisbie et al, 1999a, Lohmander et al, 1999).

Several resorption markers of articular cartilage aggrecan have been evaluated in biological fluids. Sulfated glycosaminoglycan (sGAG) is a common marker that can be used as a synthetic or degradative marker of aggrecan (i.e. an aggrecan turnover biomarker) but since its levels have been found to be high in several joint diseases, this marker is often considered as an aggrecan degradation marker. It has been reported that the levels of sGAG were higher in the biological fluids obtained from osteoarthritic horses compared to the levels in fluids from non-affected horses (Little et al, 1990; Alwan et al, 1991).

The purpose of this study was to assess the biochemical markers of bone and cartilage in serum of exercised horses with and without osteochondral fragmentation. The following markers were assessed: COL2-

3/4C_{short} as markers of type I and II collagen degradation, **CPII** as a marker of type II collagen synthesis and **CS846** as a marker of aggrecan synthesis, **234CEQ** as a marker of type II collagen degradation, **sGAG** as a marker of general aggrecan turnover, **osteocalcin** as a bone formation marker and **CTXI** as a bone resorption marker. The levels of cleaved type I collagen(**COL1-3/4C_{short}**) were determined as the difference between the **COL2-3/4C_{short}** and **234CEQ** levels. In addition, the results of serum levels of the biomarkers described in this chapter were correlated with the results obtained for the synovial fluid levels of the biomarkers described in the second chapter.

3.2 Materials and Methods

3.2.1 Animals

Sixteen healthy 2-year-old horses were used in this study. Eleven were females and five were males (Table 1). During a 2-week acclimatization period, all horses were dewormed and vaccinated. Prior to being admitted into the study, body condition, lameness examination, carpal flexion test, and an effusion test were performed.

The horses were randomly assigned to either the OA (n = 8), or control groups (n = 8). A mid carpal joint was randomly chosen from each horse in the OA group to induce osteochondral fragmentation (OCF). Blood hematology and chemistry profiles were performed to evaluate the health status of the liver and kidneys.

3.2.2 Osteochondral fragmentation model

In order to lessen the effects of surgery itself on the results, arthroscopic surgery was performed on both intercarpal joints of each horse. Starting 8 hours before surgery, each horse was medicated with procaine penicillin (22,000 IU/kg bw IM for 3 days) and phenylbutazone (4.4 mg/kg bw *per os* for 3 days). Each horse was sedated with xylazine, anesthetized with ketamine and diazepam, and anesthesia was maintained with halothane in 100% oxygen through a semi-closed breathing system. Each horse was positioned in dorsal recumbency and the carpal joints were flexed 90°. After sterile preparation and synovial fluid aspiration, bilateral carpal arthroscopic surgery was performed in a routine manner (McIlwraith, 1990). Two portals (each 6-10mm) were made in each joint, laterally and medially. The lateral portal was placed between the common digital extensor tendon and the extensor carpi radialis tendon and it was used for inserting the arthroscope into the joint. The medial portal was placed medial to the extensor carpi radialis tendon and it was used for inserting the instruments. This portal allowed an 8-mm curved osteotome to be directed perpendicular to the articular cartilage surface of the radial carpal bone at the level of the medial synovial plica. After complete arthroscopic examination of the entire joint, the osteotome was used to create an osteochondral fragment in the dorsal aspect of the distal radiocarpal bone. The fragment was allowed to remain adherent to the joint capsule proximally. A motorized arthroburr was used to debride the exposed subchondral bone between the fragment and

parent bone (Frisbie et al, 1997 and 1998). A 15 mm wide defect bed for the 8 mm wide fragment was created and the debris was not actively flushed from the joint. A 2-0 nylon suture was used to close the arthroscopic portals using simple interrupted or cruciate patterns. The limbs were bandaged and the horses allowed to recover. Each horse was kept in a 3.65 x 3.65 m stall. Bandages were changed every 3-5 days and maintained for two weeks. Sutures were removed 10 days postsurgery.

3.2.3 Experimental design

Figure 1 summarizes the experimental design used in this study. On day 0, before beginning the exercise program, blood samples were obtained from each horse through the jugular vein. Exercise consisted of running on a high-speed treadmill 5 days/week; trotting for two minutes (16-19 km/h), galloping for two minutes (32 km/h), and trotting for two additional minutes (16-19 km/h). On day 21, arthroscopic surgery was performed and the horses were rested for two weeks after surgery. The exercise started again on day 35 and continued until the end of the study (day 91). Blood samples were collected once a week from each horse beginning on day 0 and until the end of the study (day 91). The blood samples were placed into plain glass tubes, centrifuged at 3,000g for 15 minutes, and the supernatant was aliquotted in tubes and stored at -80°C for biochemical marker analysis.

3.2.4 Biomarker Assays

3.2.4.1 Bone formation marker

3.2.4.1.1 Osteocalcin

Levels of osteocalcin were measured in serum samples using a commercially available competitive immunoassay kit^I and the manufacturer's instructions were followed to perform the assay. Briefly, 25 μ L of standards, controls, or samples were added to each well of the coated 96-well plate (already coated with human osteocalcin). The plate was then incubated for two hours at room temperature after adding 125 μ L of anti-osteocalcin antibody to each well. After washing three times with wash buffer, 150 μ L of enzyme conjugate was added to each well. The plate was incubated for one hour at room temperature and then washed three times with washing buffer. After that, 150 μ L of working substrate solution was added to each well and the plate was incubated for 40 minutes at room temperature. Finally, 50 μ L of stop solution was added to each well and the optical density was read at 405 nm using an automated reader plate. The manufacturer's instructions were followed to obtain the standard curve and calculate the levels of osteocalcin in each sample (ng/mL).

3.2.4.2 Bone resorption markers

3.2.4.2.1 CTXI (CrossLaps)

The Serum CrossLaps^{II} assay, which is one step ELISA, was used to assess levels of cross-linked degradation products of the C-terminal

teloptides of type I collagen in serum samples. Assay procedure was according to the manufacturer's recommendations. Briefly, 50 μ L of the standards, controls, or samples were added to each well of the 96-well plate followed by the addition of 150 μ L of the antibody solution. After 120 minutes of incubation on a shaker at room temperature, the plate was washed five times with washing buffer. Then, 100 μ L of the substrate solution was added to each well and incubated on a shaker for 15 minutes at room temperature in the dark. After that, 100 μ L of stop solution was added to each well and the optical density was read using an automated reader plate at 450 nm. The manufacturer's instructions were followed to draw the standard curve and then calculate the levels of CTXI in each sample (ng/mL).

3.2.4.2.2 COL2-3/4C_{short}

An inhibition ELISA developed by Billingham et al (1997) was used to assess levels of COL2-3/4C_{short} in serum samples as a biomarker of both Types I and II collagen cleavage. An Immulon 96-well plate was coated with 5ng/well of the synthetic peptide used to generate the antiserum. This peptide was conjugated to keyhole limpet hemocyanin (KLH) and diluted in Tris buffer solution (TBS) at pH 7.5 and the plate was incubated overnight at 4^o C. Due to large standard deviations for recorded absorbance in outer wells, all outer wells were left uncoated and not used. After washing three times with TBS with 0.1% Tween 20 (TBS-Tween), the plate was blocked

with 100 μL /well of 1% BSA in TBS (pH 7.5) for at least 30 minutes at room temperature. The plate was then washed three times with TBS-Tween and stored at 4 $^{\circ}$ C until used for sample assay. In a round bottom polypropylene 96-well plate, 50 μL /well of standards or samples and 50 μL /well of the affinity-purified COL2-3/4C_{short} antibody R2691 diluted 1:1000 in TBS containing 1% BSA and 0.1% Tween 20 (TBS-Tween-BSA) were added. In the wells serving as blanks, 50 μL /well of TBS 1%BSA and 50 μL /well of TBS-Tween-BSA were added. In the maximum binding wells, 50 μL /well of TBS 1%BSA and 50 μL /well of antibody R2691 were added. After incubation for one hour at 37 $^{\circ}$ C, 50 μL /well of the mixture was transferred to the peptide-coated ELISA plates, and incubated for 30 minutes at 4 $^{\circ}$ C. After washing three times with TBS-Tween, 50 μL of goat anti-rabbit IgG conjugated with alkaline phosphatase and diluted 1:20,000 in TBS-Tween-BSA was added to each well. After washing four times with TBS, 50 μL of substrate^{III} was added to each well and the plate was incubated for 25 minutes at 37 $^{\circ}$ C. Then, 50 μL /well of amplifier^{III} was added and the plate was incubated for 15 minutes at 37 $^{\circ}$ C. Adding 50 μL /well of 0.3 M H₂SO₄ stopped the reaction and the optical density was read at 490 nm. A nonlinear regression equation was used to establish a sigmoidal dose response standard curve with variable slope for the determination of the concentration of COL2-3/4C_{short} in each sample ($\mu\text{g/mL}$).

The concentrations of COL1-3/4C_{short} were calculated in serum samples by subtracting the levels of 234CEQ from the levels of COL2-

$3/4C_{\text{short}}$ in each sample. This allows for an approximation of the levels of cleaved type I collagen.

3.2.4.3 Articular cartilage formation markers

3.2.4.3.1 CPII

A commercially available inhibition ELISA kit^{IV} was used to assess levels of the C-terminal propeptides of type II procollagen in serum samples. Based on the manufacturer's instructions, the assay was initiated by coating a Maxisorp ELISA 96-well plate with 100 μL /well of CPII diluted 1:100 in coating buffer and incubating the plate overnight at 4^o C. After that, 50 μL of standards or samples was added to each well of a polypropylene 96-well plate followed by adding 50 μL /well of CPII R160 antibody diluted 1:20,000 in assay buffer and pre-incubated on a shaker for 30 minutes. After washing a coated ELISA plate three times, 80 μL /well of the mixture solution in the polypropylene plate was transferred to the ELISA plate and incubated on a shaker for two hours at room temperature. After washing the plate six times, 100 μL /well of goat anti-rabbit IgG conjugated with horseradish peroxidase and diluted 1:25,000 in 6% bovine serum albumin buffer was added and the plate was incubated for 30 minutes with shaking at room temperature. After washing six times, 100 μL of TMB (3,3',5,5'-tetramethyl benzidine) substrate /well was added and the plate was incubated with shaking for 30 minutes at room temperature. Finally, 100 μL /well of stop solution/well was added and the optical density was read at 450 nm. The

manufacturer's instructions were followed to draw the standard curve and calculate the levels of CPII in each sample (ug/mL).

3.2.4.3.2 CS846

A commercially available competitive ELISA kit^V was used to assess levels of the chondroitin sulfate 846 epitope in serum samples. Based on the manufacturer's instructions, this assay began by coating an ELISA 96-well plate with 100 µL/well of goat anti-mouse IgM diluted 1:500 in coating buffer and incubating overnight at 4^o C. After washing three times, the following were added to the plate: 50 µL/well of biotin-PG conjugate diluted 1:5000 in 6% BSA, 50 µL/well of PG standards or samples, and 50 µL/well of mouse anti-PG 846 antibody diluted 1:10,000 in assay buffer. After incubating on a shaker for two hours at room temperature and washing six times, 100 µL of streptavidin horseradish peroxidase conjugate diluted 1:2500 in 6% BSA was added to each well. After incubation on a shaker for 30 minutes at room temperature and washing six times, 100 µL of substrate was added to each well. After further incubation for 30 minutes, 100 µL/well of stop solution was added and the optical density was read at 450 nm. The manufacturer's instructions were followed to create the standard curve and then calculate the levels of CS846 in each sample (ng/mL).

3.2.4.4 Articular cartilage resorption markers

3.2.4.4.1 234CEQ

An inhibition ELISA developed by Billinghamurst et al (2001) was used to assess the levels of the 234CEQ neoepitope in serum samples. An Immulon 96-well plate was coated with 5ng/well of the synthetic peptide used to generate the 234CEQ antiserum. This peptide was diluted in Tris buffer solution (TBS) at pH 7.5 and incubated overnight at 4^o C. Due to large standard deviations in absorbance in outer wells, they were left uncoated and not used in the assay. After washing three times with TBS 0.1% Tween 20 (TBS-Tween), the plate was blocked with 100 µL/well of 1% BSA in TBS (pH 7.5) for at least 30 minutes at room temperature. After that, the plate was washed three times with TBS-Tween and stored at 4^o C. In a round bottom polypropylene 96-well plate, 50 µL/well of standards or samples and 50 µL/well of the affinity purified 234CEQ antibody 5373 diluted 1:1000 in TBS containing 1% BSA and 0.1% Tween 20 (TBS-Tween-BSA) was added. In the blank wells, 50 µL/well of TBS-Tween-BSA was added. In the maximum binding wells, 50 µL/well of TBS 1%BSA and 50 µL/well of antibody 5373 (234CEQ antiserum) were added. After incubating for one hour at 37^oC, 50 µL/well of the mixture was transferred to the coated ELISA plate, and incubated for 30 minutes at 4^oC. After washing three times with TBS-Tween, 50 µL of goat anti-rabbit IgG conjugated with alkaline phosphatase and diluted 1:20,000 in TBS-Tween-BSA was added to each well, and the plate was incubated for one hour at 37^oC. After washing four times with TBS, 50 µL of substrate^{III} was added to each well and incubated for 25 minutes at 37^oC. After that, 50 µL/well of amplifier^{III} was added and

incubated for 20 minutes at 37 °C. The reaction was stopped by adding 50µL/well of 0.3 M H₂SO₄ and the optical density was read at 490 nm. A nonlinear regression equation was used to establish a sigmoidal dose response standard curve with variable slope for the determination of the concentration of 234CEQ in each sample (ug/mL).

3.2.4.4.2 sGAG

A modified 1,9-dimethylmethylene blue (DMMB) dye-binding assay was used to determine GAG concentration in serum samples (Carroll, 1987, 1989). Initially, 100 µL of each sample was placed in a tube and 100 µL of the digestion enzyme papain solution was added to each tube. This digestive papain enzyme solution was prepared by adding 32.81 gm sodium acetate and 3.72 gm disodium EDTA to double distilled water (ddH₂O) for a final volume of 1 liter, and immediately before use, 2.42 mg cysteine and 20 µL of papain suspension were added to each 1 mL of the digestion solution. After incubating the mixtures of sample and papain solution for 18 hours at 60 °C, the reaction was stopped by adding 4 µL of iodoacetamide to each tube. The stop solution can be prepared by adding 0.925 gm of iodoacetamide and ddH₂O to a final volume of 25 mL. After that, 10 µL of controls, standards, or samples were placed in duplicate in a U-bottom 96-well plate and then 190 µL of dye solution was added to each well. The optical density was read at 530 nm. To prepare one liter of dye solution, 3.04 gm of glycine, 2.37 gm sodium chloride and ddH₂O were put in a flask. After protecting the flask

from the light, 16 mg of DMMB was added and stirring continued overnight. The levels of sGAG in each sample (mg/mL) were determined based on a regression equation used to draw a standard curve.

3.2.5 Statistical Analysis

Data normality was checked using plots of residual versus predicted values. If normality assumption was not met, the data was transformed using natural logarithm (log) and normality reverified. In cases where log transformations were required, statistics analysis was performed on the transformed data.

Both the effects of exercise and group were determined in this study. A split plot design with repeated measures analysis was used as the statistical model to evaluate the dependent variables as they pertain to the serum samples. In the SAS program, a mixed model using Proc Mixed was utilized to do the statistical analysis. In this model, the dependent variables included the levels of osteocalcin, and CTXI CPHI, COL2-3/4C_{shorts}, COL1-3/4C_{shorts}, CS846, 234CEQ and sGAG. The independent variables in this model were day of sample collection and the presence or absence of a chip in the horse. The subject within-treatment variables was used as a random effect variable. A general linear model procedure was used to perform a multivariate analysis of variance (ANOVA) analyzing independent variables for both the main and the interaction effects on the dependent variables. The highest interaction with $P < 0.05$ was considered the most significant and was reported. When there was a significant interaction effect, a Least Square

Means test was used to make individual comparisons. A Pearson correlation coefficient analysis was used to study the relationship among the dependent variables. In addition, a Pearson correlation analysis was used to compare the levels of synovial fluid biomarkers (chapter II) with those in the serum. Values of the correlation coefficients were classified into four grades: slight ($r \leq 0.25$), mild ($0.25 < r \leq 0.50$), moderate ($0.50 < r \leq 0.75$), and strong ($0.75 < r \leq 1$) correlations. The significance level was set at $P < 0.05$.

3.3 Results

3.3.1 Animals

No prior lesions were noted in any joints during arthroscopic surgery except in one horse, which had partial tearing of the medial palmar intercarpal ligament in both intercarpal joints. Since no clinical signs were observed due to this lesion, this horse was not excluded from the study. For all horses, recovery from anesthesia was smooth and none required additional analgesia beyond three days of phenylbutazone administration. There were no significant differences between males and females for the serum levels of biomarkers.

3.3.2 Biomarkers

3.3.2.1 Bone formation marker

3.3.2.1.1 Osteocalcin

Figure 2 shows the mean levels of serum osteocalcin during each sampling period for each group. We found significant differences between

groups in the levels of serum osteocalcin only at the end of the study (day 91) ($P < 0.001$). Over time starting on day 42, there was a significant increase in the concentration of serum osteocalcin as a result of exercise ($P < 0.05$).

3.3.2.2 Bone resorption markers

3.3.2.2.1 CTXI

Figure 3 shows the levels of serum CTXI during each sampling period for each group. It was found that there was no significant difference between groups in the concentration of serum CTXI ($P > 0.1$). However over time, there was a significant change in the concentration of serum CTXI due to exercise ($P < 0.05$). The serum CTXI levels fluctuated with the highest levels on days 21 and 63 and the lowest level on day 35.

3.3.2.2.2 COL2-3/4C_{short}

Figure 4 shows the levels of COL2-3/4C_{short} in serum during each sampling period for each group. The group and time had effects on the concentration of serum COL2-3/4C_{short} ($P < 0.01$). In addition, there were significant interactions between the group and the time variables ($P < .001$). The differences between groups in the levels of serum COL2-3/4C_{short} were first seen three weeks postsurgery. However, the significant differences were only in the last two weeks of the study period (Figure 4). There was a strong correlation between COL2-3/4C_{short} and CS846 ($r^2 = 0.64$), COL2-3/4C_{short} and sGAG ($r^2 = 0.64$), and a moderate correlation between COL2-3/4C_{short} and CPH ($r^2 = 0.35$) and between COL2-3/4C_{short} and osteocalcin ($r^2 = 0.29$).

3.3.2.2.3 COL-13/4C_{short}

The time, group and time and group interactions showed significant effects on the levels of COL1-3/4C_{short} ($P < 0.001$). The levels of COL1-3/4C_{short} were significantly higher in the OA group compared to the control group during only the last two weeks of the study (day 84 and 91). Over time, there was an increase in the levels of COL1-3/4C_{short} between weeks (Figure 5).

3.3.2.3 Articular cartilage formation markers

The serum concentrations of CII and CS846 are shown in Figures 6 and 7, respectively. Exercise had a slight effect on the levels of serum CII and CS846. Group had a significant effect ($P < 0.01$) on the levels of these markers. The significant increases in the levels of CII were first seen three weeks post surgery (on day 42) and continued to be significant until the end of the study (Figure 6). In the case of CS846, the first significant difference between the two groups was seen two weeks post-surgery (on day 35) and this continued until the end of the study (Figure 7). A relatively strong correlation between serum CII and CS846 was found ($r = 0.77$).

3.3.2.4 Articular cartilage resorption markers

3.3.2.4.1 234CEQ

Figure 8 shows that over time, there was an increase in the concentration of 234CEQ ($P = 0.02$). With exercise, the levels of serum

234CEQ increased and then decreased during the resting period. Upon the resumption of exercise, the levels of 234CEQ started to increase again (Figure 8). In addition, there was a trend towards an increase in the levels of 234CEQ for the OA group compared to the control group starting one week post-surgery, however this increase did not reach significance ($P = 0.08$).

3.3.2.4.1.1 sGAG

Figure 9 shows the concentrations of sGAG in the sera of the horses. Time, group and the interaction of group and time showed significant effects ($P < 0.001$) on the levels of serum sGAG. Over time, there was a significant increase between weeks in the levels of sGAG. The significant differences between groups were first seen four weeks post-surgery and continued until the end of the study (Figure 9). Furthermore, there were correlations between serum sGAG and various serum markers including CS846 ($r = 0.93$), CPII ($r = 0.68$), COL2-3/4C_{short} ($r = 0.81$), and osteocalcin ($r = 0.66$).

3.4 Correlations between serum and synovial fluid biomarkers

Table 2 shows the Pearson correlation coefficients between the levels of markers in synovial fluid and serum. The levels of osteocalcin, COL2-3/4C_{short}, and COL1-3/4C_{short} in serum from the control group were 10%, 3 fold and 16 fold, respectively, higher than their mean levels in synovial fluid from the control group (mean levels of right and left joints). The levels of osteocalcin, COL2-3/4C_{short}, and COL1-3/4C_{short} in serum from the OA group were 10%, 3 fold and 9 fold, respectively, higher than their average levels in

synovial fluid from the OA group. On the other hand, the mean levels of CPII, 234CEQ, CS846, and sGAG in synovial fluid from the control group were 50%, 10 fold, 20 fold, and 3 fold, respectively higher than their levels in serum from the control group. The levels of CPII, 234CEQ, CS846, and sGAG in synovial fluid from the OA group were 70%, 23 fold, 22 fold, and 3 fold, respectively, higher than their average levels in serum from the OA group.

3.5 Discussion

In this study, the concentrations of cartilage and bone markers have been measured in serum. Blood is easy to obtain, sampling can be repeated often, and blood has a relatively constant volume in the body (i.e. independent of joint flux). The other advantage of measuring the biomarkers in blood is the usefulness for evaluating systemic changes. In contrast, measuring biomarker levels in blood has some disadvantages over other fluids such as synovial fluid. The concentrations of some markers in blood are below the detection limit of their assays. Both liver and kidney functions may have a significant effect on the levels of markers in blood (Thonar et al, 1993). Measuring markers in blood likely has less benefit compared to synovial fluid when studying changes in a single joint.

Subchondral bone changes have been thought by some to play a major role in the pathogenesis of OA. One potential way to detect the changes in the subchondral bone is by measuring the concentrations of molecular markers of bone synthesis and resorption in body fluids. In this

study, osteocalcin was measured in serum as a bone formation marker whereas CTXI and COL1-3/4C_{short} were assessed as bone resorption markers. In addition, the levels of serum COL2-3/4C_{short}, which represents the levels of cleaved types I and II collagens, were measured.

In this study, we found that the concentrations of osteocalcin were significantly affected by exercise and OA. We found that exercise caused an increase in the levels of osteocalcin from week to week starting on week six (Figure 2). This suggests that exercise alone stimulated bone formation in horses. Several studies in humans and horses have found similar results. Maenpaa et al (1988) have found that the levels of serum osteocalcin decreased in foals kept in stalls compared to when they were at pasture. In humans, a positive correlation between exercise and bone formation has been reported (Fujimura et al, 1997), and the level of osteocalcin was reported to be 50% higher in exercised compared to non-exercised men (Bell et al, 1988). In our study, the significant increase in the levels of osteocalcin due to exercise was first seen on day 42 after the beginning of exercise. This is similar to what has been reported in humans (Fujimura et al, 1997; Eliakim et al, 1997). The exact mechanism(s) of how exercise stimulates bone synthesis is unknown, but the stress on bone due to exercise might lead the bone cells to accommodate that by increasing the numbers and activities of osteoblasts, and this in turn leads to increases in bone synthesis and osteocalcin levels. There are studies that contradict what we found in our study. Kawcak et al (1998) have reported that there were no significant

differences in the levels of serum osteocalcin between treadmill-exercised and handwalked horses over time. In the Kawcak study, serum samples were collected monthly, and human radioimmunoassay kits were used to measure the levels of osteocalcin in serum samples. In our study, serum samples were collected weekly, and an ELISA was used to measure the levels of osteocalcin. In each group, 6 horses were used in Kawcak study whereas 8 horses were used in each group in our study. This suggests that the power of the study was higher in our study compared to Kawcak study. In addition, since handwalking was used for the horses of the control group in the Kawcak study, this might indicate that handwalking itself is enough to stimulate bone formation. Therefore, no significant differences would likely be found between the two groups in that study.

We found that OA had an effect on the concentration of serum osteocalcin. The significant differences between the two groups were found on the last week of the study (Figure 2). Therefore, the significant increase in bone mineralization appears to have occurred ten weeks post-surgery. The levels of serum osteocalcin obtained from exercised horses with OCF were 43% higher than those from the exercised-only horses. Gevers et al (1988) have reported that there was a significant increase in the serum levels of osteocalcin in postmenopausal women with generalized OA compared with age- and sex-matched controls. It has been shown in another study that there was an increase in serum osteocalcin content in iliac crest bone from patients with OA, corresponding to a shift towards higher densities in the

mineralization profile (Raymaekers et al, 1992). On the other hand, Garnero et al (2001) have reported that total serum osteocalcin (measuring intact and N-mid-peptide fragments of osteocalcin) decreased in patients with knee OA compared with normal controls. However, serum samples from elderly people with chronic OA were used in Garnero's study (2001). Therefore, the results of this study cannot be compared with our study. Our results suggest that exercise has a rapid stimulating effect on bone formation whereas osteochondral fragmentation produces significant increases in detectable bone formation several weeks after the injury.

The levels of CTXI were assessed in serum as a bone resorption marker. We found that exercise positively affected the concentration of serum CTXI and a fluctuating curve was obtained over time peaks at days 21 and 63. The reason for these two peaks was possibly due to training the horses in the beginning, then resting them for two weeks before resuming exercise. After the second peak on day 63, there was a decrease in the levels of CTXI. Starting on day 70, it seems that the levels of bone resorption adapted to the levels of exercise that the horses had. This suggests that the first month of exercise is the most critical time for bone resorption in the two-year old horses that enrolled in our exercise program. Therefore, more attention should be given to the horses and to the type of exercise during that time. There are few studies that have assessed the levels of CTXI in the biological fluids of humans. Jaffre et al (2001) have reported that there was a significant increase in the levels of CTXI in sera of young female gymnasts

compared to the non-gymnast controls and concluded that gymnastics stimulates bone resorption activity.

There was no significant difference in the concentrations of CTXI between the two groups in our study. Therefore during the period of study, we found that CTXI was not a useful biomarker to detect the increase of bone resorption in the OA group. In human studies, Garnero et al (2001) have reported that there was a significant decrease in the levels of both serum and urine CTXI in the patients with knee OA compared with normal healthy patients. However in their study, all patients had chronic OA.

The levels of serum COL2-3/4C_{short} which measures the levels of collagenase-cleaved types I and II collagens were assessed in this study. We found that exercise and OA influenced the levels of serum COL2-3/4C_{short} in horses. Exercise had positive effects on the levels of serum COL2-3/4C_{short} starting from the first week of the study until the end of the study. This suggests that exercise stimulates the cleavage of type I and II collagens.

Starting on day 42, there was an increase in the levels of serum COL2-3/4C_{short} obtained from OA group compared to that from the normal controls. However, significant differences were only observed in the last two weeks (days 84 and 91). This suggests that OA stimulates the cleavage of type I and II collagen by collagenases in exercised horses. The levels of serum COL2-3/4C_{short} from exercised horses with OA were 3 fold higher than those from the exercised-only horses. Very few studies have assessed the body fluids levels of COL2-3/4C_{short}. Billingham et al (1997) have found

that there was a significant increase in the levels of COL2-3/4C_{short} in osteoarthritic compared to nonarthritic cartilage. Since COL2-3/4C_{short} measures the levels of cleaved types I and II collagens together, and 234CEQ (see below) measures type II collagen, it is possible to calculate the levels of type I collagen (COL1-3/4C_{short}) by subtracting the levels of 234CEQ from the levels of COL2-3/4C_{short}. The strong correlation that we found ($r^2 = 0.90$) between COL2-3/4C_{short} and the calculated COL1-3/4C_{short} increased our confidence in using this method for determining the concentrations of COL1-3/4C_{short}. When analyzing the calculated data for COL1-3/4C_{short}, we found that there were significant effects of time, group, and time and group interactions ($P < 0.01$) on the levels of COL1-3/4C_{short}. The significant differences between groups were seen in the last two weeks of the study (Figure 5). This suggests that exercise alone has a rapid stimulating effect on type I collagen resorption whereas osteochondral fragmentation produced detectable type I collagen cleavage several weeks post-injury. The levels of serum COL1-3/4C_{short} obtained from exercised horses with OCF were 2 fold higher than those from the exercised-only horses. This study showed that COL1-3/4C_{short} was more sensitive to detect type I collagen cleavage than CTXI. Thus, measuring COL13/4C_{short} is more sensitive in detecting bone resorption than measuring CTXI in this model.

Collagen, particularly type II, is a major structural component of the extracellular matrix of articular cartilage. CII and 234CEQ are two markers that are used to estimate collagen synthesis and degradation,

respectively (Nelson et al, 1998; Billinghamurst et al, 2001). We found that the concentrations of CPII significantly increased in the sera of the OA group compared to the normal control group (Figure 6). This significant increase was seen three weeks postsurgery and continued until the end of the study. This suggests that OCF significantly stimulated the synthesis of type II collagen. The levels of CPII in the sera of the OA group were 50% higher than those from the control group.

A study in horses has shown a significant increase in levels of serum CPII for horses with OCF compared to control horses (Frisbie et al, 1999). In humans, serum CPII was significantly higher in patients with various stages of OA (except in the most advanced phases) compared to the levels in humans without disease (Lohmandar et al, 1996).

Exercise had direct effects on the serum levels of 234CEQ starting from the first week of the study until the end of the study. This suggests that exercise stimulated the cleavage of type II collagen. We failed to find significant differences in the sera levels of 234CEQ between the two groups even though there was a trend towards an increase in the levels for the OA group compared to the control group (Figure 8). The levels of 234CEQ in the sera of the OA group were 20% higher than those from the control group. Billinghamurst et al (2001b) have reported that cartilage from an osteoarthritic joint of a horse had increased staining with the 234CEQ antibody compared to nonarthritic cartilage.

Proteoglycans, as aggrecan molecules, are considered to be another major structural component of articular cartilage. CS846 and sGAG are two markers that reflect proteoglycan synthesis and turnover, respectively (Alwan et al, 1991; Rizkalla et al, 1992; Frisbie et al, 1999). We found in this study that exercise and OA both significantly increased the serum levels of CS846 and sGAG.

Exercise and group significantly increased the levels of CS846 in serum (Figure 7). With exercise, the serum levels of CS846 significantly increased between weeks. This suggests that exercise stimulates aggrecan synthesis. To the best of our knowledge, this is the first report describing the effects of exercise on the concentration of serum CS846 in horses.

The concentration of serum sGAG increased with exercise during the study (Figure 9). This suggests that exercise alone may stimulate increased proteoglycan turnover in healthy horses. In a recent study, Murray et al (2001) have reported that the content of sGAG in cartilage from horses undergoing high intensity training was significantly higher than in cartilage from horses undergoing low intensity training. Brama et al (1999) have reported that sGAG content increased with increasing exercise in five-month-old foals and younger, but this increase disappeared after a further 6 months of similar exercise. This finding is comparable with our study because the study period was shorter in our study (3 months) compared to Brama study (> 6 months). Also, in our study two-year old horses were used

whereas in Brama study young foals predisposed to osteochondrosis were used.

Of the biomarkers assessed in this study, CS846 and sGAG levels in serum provide the earliest information concerning the presence of OA. The significant differences in serum levels of CS846 and sGAG between the two groups started two weeks and four weeks, respectively post-surgery (Figures 7 and 9). This finding suggests that early OA significantly stimulated the levels of aggrecan synthesis and turnover. However, the stimulation of aggrecan synthesis preceded its degradation in early OA. Therefore, aggrecan synthesis and not degradation markers may represent the first changes seen in early OA. This study also suggests that aggrecan degradation is not the stimulus for aggrecan synthesis at least in the very early stages of OA, and the mediator(s) that stimulate aggrecan synthesis are likely released earlier than those that stimulate aggrecan degradation. The average levels of CS846 and sGAG in the sera of exercised horses with OA were 1 fold and 60% higher than those from the exercised only horses.

Studies in humans and horses with joint diseases have shown an increase in body fluid levels of CS846 epitope with disease (Rizkalla et al, 1992; Mansson et al, 1995; Bassleer et al, 1996; Frisbie et al, 1999; Ishigurom et al, 1999; Lohmander et al, 1999). Frisbie et al (1999) found significantly higher levels of CS846 in synovial fluid and serum from horses with OCF compared to the levels in fluid from unaffected horses.

The effects of OA on the concentrations of sGAG in OA fluids have been evaluated in humans and horses. Alwan et al (1991) used the DMMB assay to assess the levels of sGAG in synovial fluid, sera, and urine from horses with OA. They found that the concentrations of sGAG in these fluids from osteoarthritic horses were generally higher than levels in fluids from non-arthritic horses and they were also higher than levels in fluids from horses with other types of joint disease (Alwan et al, 1991). In fact, the serum levels of sGAG were significantly higher for OA joints than normal, septic and joints with osteochondritis dissecans. It was concluded that synovial fluid sGAG couldn't be considered a specific diagnostic marker for OA because its concentration was also increased although not significantly with other types of arthritis (Alwan et al, 1991). Little et al (1990), using a modified alcian blue precipitation method, also found that the concentration of sGAG in synovial fluid from osteoarthritic joints was significantly higher than non-arthritic controls. However, the concentration of sGAG did not correlate with the degree of articular cartilage erosion. It should be noted that sGAG in the biological fluids comes from both the degradation and synthesis of aggrecan. However, sGAG is considered an aggrecan degradation marker because its levels were found to be high with the arthritic diseases that associated with articular cartilage destruction.

A strong positive correlation between CS846 and sGAG in serum ($r=0.93$) was found in this study. Therefore it can be concluded that during

exercise and in early OA, the increase in aggrecan degradation is associated with an increase in aggrecan synthesis.

Since there were early significant differences in the serum sGAG levels between groups, and since we did not find significant difference in the serum levels of 234CEQ between groups, it may be assumed that aggrecan degradation precedes collagen cleavage in articular cartilage in this model. Therefore, this study supports the hypothesis that suggests that proteoglycan depletion precedes collagen breakdown in early OA (McIlwraith and Van Sickle, 1981; Billingham et al, 2000). Since the significant differences in serum CS846 levels between groups occurred earlier than that for CPII levels, it can be assumed that increases in aggrecan synthesis precedes increases in collagen synthesis in articular cartilage. Since aggrecan turnover preceded collagen turnover and since aggrecan synthesis preceded degradation, we hypothesize that changes in aggrecan synthesis is the first that can be detected in early OA with biomarkers. We found that significant collagen synthesis occurred earlier than collagen degradation, and there was a mild correlation between the serum levels of CPII and 234CEQ ($r = 0.36$). This might indicate that collagen synthesis is not stimulated by collagen degradation. We also found that there was a strong positive correlation between CS846 and CPII ($r = 0.77$) and between sGAG and COL2-3/4C_{short} ($r = 0.81$). This suggests that some early mediator(s) might stimulate aggrecan and collagen synthesis at the same time and some mediator(s) might stimulate aggrecan and collagen degradation at the same time.

However, the aggrecans are much more sensitive to these mediator(s) and faster to be degraded and synthesized compared to collagen.

It has been reported that aggrecanases and matrix metalloproteinases (MMPs) including stromelysins are the major enzymes that degrade proteoglycans (Galloway et al, 1983; Sanday et al, 1992; Lohmandar et al, 1993; Lark et al, 1995) whereas other MMPs including collagenases and gelatinases are the major enzymes that cleave collagens in articular cartilage (Lohmandar et al, 1993 and 1995; Clegg et al, 1997; Brama et al, 1998). In our study, the significant increase in the levels sGAG, COL2-3/4C_{short} and COL1-3/4C_{short} in the OCF group might suggest that there were increases in the levels and activities of the aggrecanases and collagenases. Other studies have found that the levels and activities of these enzymes increase in the osteoarthritic cartilage (Dean et al, 1987; Clegg et al, 1997; Brama et al, 1998). Lohmandar et al (1996) have found a positive correlation between CPII, CS846, and MMP-3 in synovial fluid. Therefore, these degradative enzymes are probably the earlier mediators in aggrecan and collagen degradation.

In this study we found that the changes in the concentrations of proteoglycan and type II collagen markers preceded the changes in the levels of bone markers in our OA model. Therefore, this study supports the hypothesis suggesting that articular cartilage changes precede bone changes in early OA.

Correlations between the serum and synovial fluid levels of the biomarkers were determined in this study. In general, the concentrations of osteocalcin are expected to be higher in sera than in synovial fluids. However, our study showed that the levels of serum osteocalcin were only 10% higher than levels in the synovial fluid. Several reasons could explain the source of osteocalcin assessed in the synovial fluid. Osteocalcin has a small molecular weight and it might enter the joint from the serum. Osteocalcin might be produced locally in the joint, or it might be produced by the subchondral bone and then enters the joint cavity. Since we found that there was a relatively low correlation between serum and synovial fluid samples and since the significant differences between groups were seen on day 91 for both fluids, we think that part of osteocalcin in synovial fluid might come from the serum and the other part might be produced locally in the joint. Since the significant differences between the OA and control groups were seen only on day 91 in both serum and synovial fluid samples, serum samples can replace the synovial fluid samples in assessing the levels of osteocalcin.

Few studies have compared serum and synovial fluid levels of osteocalcin in humans with OA (Campion et al, 1989; Mattei et al, 1992; Salisbury and Sharif, 1997). These studies have found that the levels of osteocalcin in normal and osteoarthritic patients were lower in synovial fluid than serum. The levels of synovial fluid osteocalcin were directly correlated with the serum levels (Salisbury and Sharif, 1997). However in the Campion

study, four patients (n = 30) had higher levels of osteocalcin in synovial fluid than in serum (n=30) (Campion et al, 1989). This led the author to suggest that there were local production or release to the osteocalcin in the joint (Campion et al, 1989).

The levels of the types I and II collagen degradation markers were strongly correlated between serum and synovial fluid. Ninety percent was the correlation between serum COL2-3/4C_{short} in the OA group and that in synovial fluids from joints with OCF. The levels of COL2-3/4C_{short} were 3 fold higher in serum compared to synovial fluids. In addition to bone, type I collagen is found in other articular tissues including tendons and ligaments, and obviously the structures of these tissues are affected by exercise and/or OCF. Furthermore, type I collagen is found in other tissues including skin and many interstitial connective tissues. Exercise and/or OCF might stimulate collagen degradation in all these tissues. The significant differences between groups in the levels of synovial fluid and serum COL2-3/4C_{short} were first seen five, and nine weeks post-surgery, respectively. Although the levels of serum COL2-3/4C_{short} were 3 fold higher than levels in synovial fluid, the latter was more sensitive than the former because the significant differences in the levels of COL2-3/4C_{short} in synovial fluid appeared four weeks before these identified in serum. The reason for this may be that the level of exercise used in this study could not rapidly stimulate type I collagen resorption from tissues other than bone (e.g. skin and tendons) or it takes that long for changes to be seen in serum (i.e. levels to build up).

The correlation in the levels of COL1-3/4C_{short} between serum and synovial fluid samples from the OA groups was found to be relatively strong. The levels of COL1-3/4C_{short} were 9 fold higher in serum compared to synovial fluids. Interestingly, the significant differences between OA and control groups in the levels of COL1-3/4C_{short} were first seen five and nine weeks post-surgery in synovial fluid and serum samples, respectively. Therefore, significant changes in type I collagen degradation may be detected earlier in synovial fluid than serum. The reason for this may be because the effect of the level of exercise that we used in this study in producing detectable degradation in type I collagen of tissues other than bone (e.g. skin, tendons) is slower than that in bone or it takes that long for detectable differences in the serum.

The levels of 234CEQ were 23 and 10 fold higher in synovial fluid compared to levels in serum between the serum and synovial fluid in OA and control groups, respectively. This is an easily explained finding because 234CEQ neoepitope is a marker used for determining type II collagen degradation (Billingham et al, 2001b), which increases with exercise and/or OCF in horse joints. Since the significant differences in the levels of 234CEQ between the OCF and the control groups were noted five weeks postsurgery in synovial fluid, and since no significant difference was found in serum 234CEQ between groups, it can be concluded that synovial fluid levels of 234CEQ is a good indicator of increased type II collagen degradation. The serum was not sensitive enough to be used as a replacement to the synovial

fluid in measuring the levels of ²³⁴CEQ. The relatively lower correlation between the synovial fluid and serum ²³⁴CEQ suggests that further degradation of ²³⁴CEQ might occur in the lymphatic system as well as in the blood stream.

The correlation between the serum and synovial fluid levels of CPII was relatively strong especially between the OA groups for the two fluids. Our study showed that approximately 50% of the synovial fluid CPII entered the blood stream. Since the levels of synovial fluid CPII were higher than its levels in serum, this suggests that either the concentration of marker is diluted when entering the blood or it might degraded in the lymphatic system and/or after entering the blood stream. In both synovial fluid and serum, the significant differences between groups in the levels of CPII were first seen three weeks postsurgery. This suggests that measuring the serum levels of CPII is a valuable method that can be used as a replacement for measuring the synovial fluid levels of CPII to assess type II collagen synthesis in horses with OCF.

The serum levels of CS846 were positively correlated with its levels in synovial fluid. The levels of CS846 were 20 fold higher in synovial fluid than in serum. This suggests that the levels of marker are diluted in blood or further degradation for this marker may occur in the lymphatic system and after entering the blood stream. Other studies in humans and horses have found the same result (Pool et al, 1994; Frisbie et al, 1999a). The significant differences between groups in the levels of synovial fluid and serum CS846

were seen one week and two weeks postsurgery, respectively. This suggests that both fluids are highly sensitive in detecting changes in proteoglycan synthesis between groups, but synovial fluid is more sensitive than the serum.

A strong positive correlation was found between serum and synovial fluid sGAG. The significant differences between groups in the synovial fluid and serum levels of sGAG were first seen two and four weeks postsurgery, respectively. The levels of sGAG were 3 fold higher in the synovial fluid than in the serum. Therefore, the synovial fluid is more sensitive than the serum in detecting proteoglycan degradations. Alwan et al (1991) have reported that the serum levels of sGAG were lower than those in synovial fluid. However, no correlation was found between serum sGAG levels and those in the synovial fluid in horses with OA (Alwan et al, 1991). The results in Alwan study (1991) were different from what we found in our study. This might be because in Alwan study (1991), synovial fluid samples were collected from different joints mainly the carpal and the fetlock joints of non-exercised-horses with different ages. From each joint, only one synovial fluid sample was collected. In Alwan study (1991), the joints already had OA in different degrees of severity and therefore the range of synovial fluid levels of sGAG was very broad (from 25 to 600 ug/mL). In addition, most of the cases in Alwan study were under treatment. All these reasons might be responsible for the different results between our study and the Alwan study (1991).

In summary, positive correlations were found between the serum and synovial fluid levels of articular cartilage and bone biomarkers. In general, the levels of articular cartilage biomarkers were higher in synovial fluid than serum and the levels of bone biomarkers were higher in serum than synovial fluid. Evaluating the levels of aggrecan turnover markers in serum appears to be a sensitive way to detect changes among groups in terms of aggrecan metabolism. Evaluating the levels of collagen degradation biomarkers detected changes earlier in synovial fluid compared to serum samples. The serum was a useful fluid to evaluate the biomarker levels of type II collagen synthesis and bone formation.

Overall, this study measured the levels of bone and articular cartilage metabolic markers in serum samples obtained from exercised horses with or without experimentally-induced OA. Both exercise and OA stimulated cartilage and bone metabolism. Our study showed that changes in articular cartilage can be detected by measuring fluid levels of biomarkers several weeks before detecting changes in bone metabolism in equine joints with early OA. Within articular cartilage, increases in aggrecan turnover were detected before collagen turnover. Within aggrecan, aggrecan synthesis was detected earlier than its degradation.

3.6 Tables and Figures

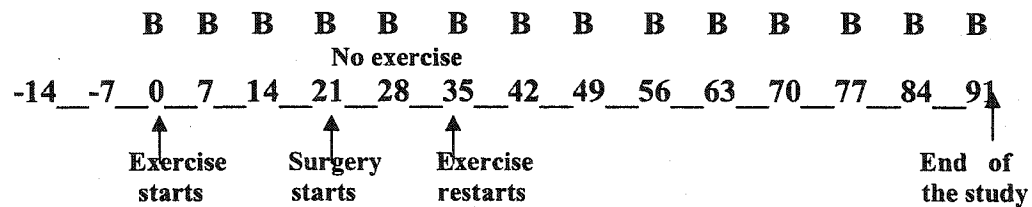


Figure 1. The timeline of this study is summarized in this figure. The study starts two weeks before the beginning of the exercise (-14) and finished on day 91. On day 21, surgery was performed for each horse. After that, the horses were rested for two weeks. Then, the horses reexercised and continued exercising until the end of the study. Serum samples were collected once a week from each horse beginning on day 0 and until the end of the study (day 91). B = blood sample.

Table 1. The gender of the horses in each group.

	Male Horses	Female Horses
OCF Group	3	5
Control Group	2	6

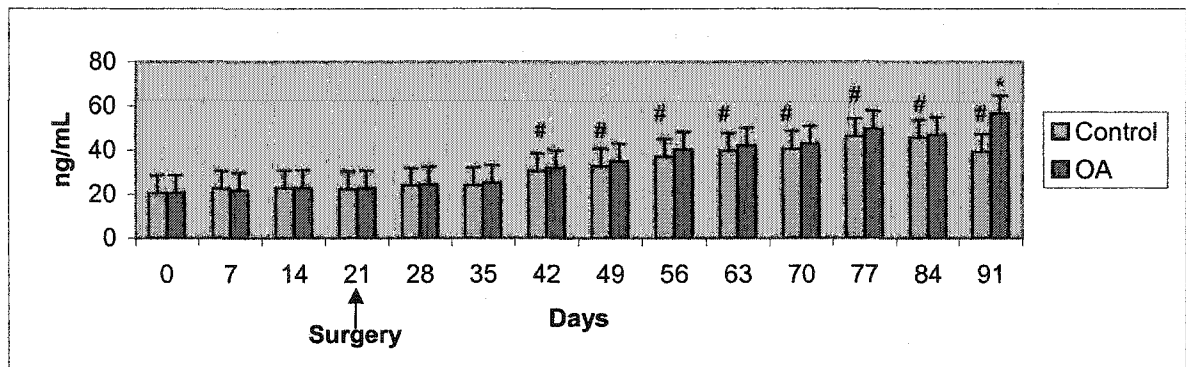


Figure 2. The mean ($\pm$ SEM) concentration of osteocalcin in serum. Starting on day 42, the levels of osteocalcin increased with exercise and continued increasing until the end of the study. On day 91, the levels of osteocalcin were significantly higher in the OA group compared to the control group. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of osteocalcin compared to day 0 in the control group ($P < 0.05$).

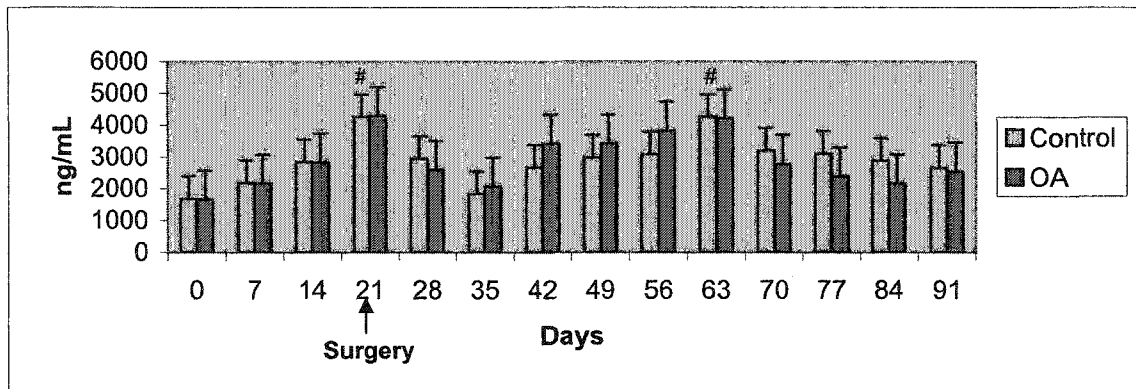


Figure 3. The mean (± SEM) concentrations of CTX in serum. There was no significant difference between the two groups at any time point. The # indicates the days that significantly have higher levels of CTX compared to day 0 in the control group ($P < 0.05$).

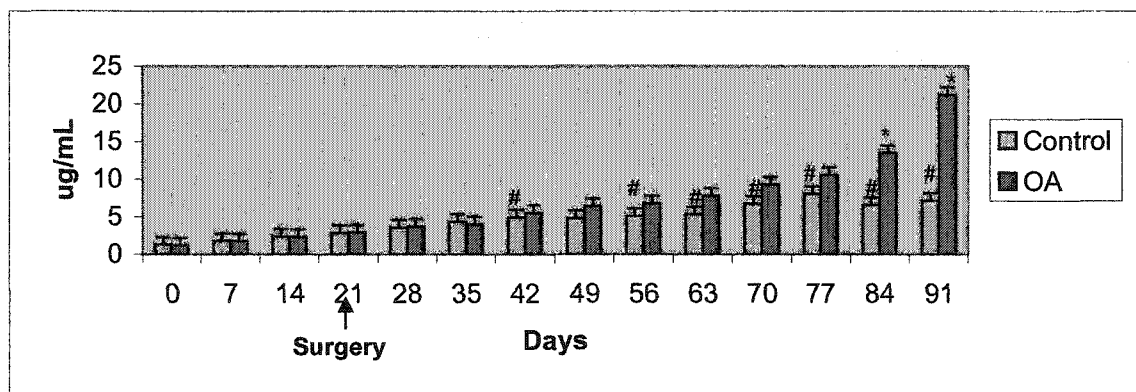


Figure 4. The mean (± SEM) concentration of COL2-3/4C_{short} in serum. The levels of COL2-3/4C_{short} increased with exercise and continued increasing until the end of the study. On days 84 and 91, the levels of COL2-3/4C_{short} were significantly higher in the OA group compared to the control group. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of COL2-3/4C_{short} compared to day 0 in the control group ($P < 0.05$).

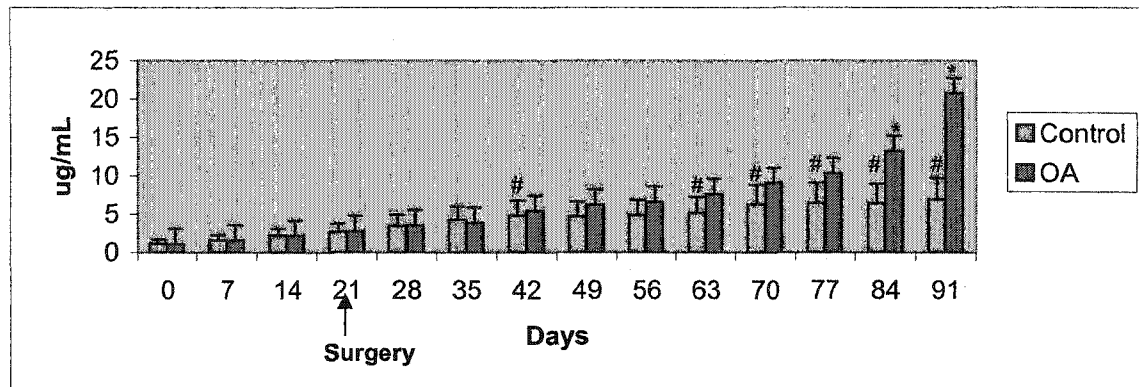


Figure 5. The mean ($\pm$ SEM) concentration of COL1-3/4C_{short} in serum. The levels of COL1-3/4C_{short} increased with exercise and continued increasing until the end of the study. On days 84 and 91, the levels of COL1-3/4C_{short} were significantly higher in the OA group compared to the control group. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of COL1-3/4C_{short} compared to day 0 in the control group ($P < 0.05$).

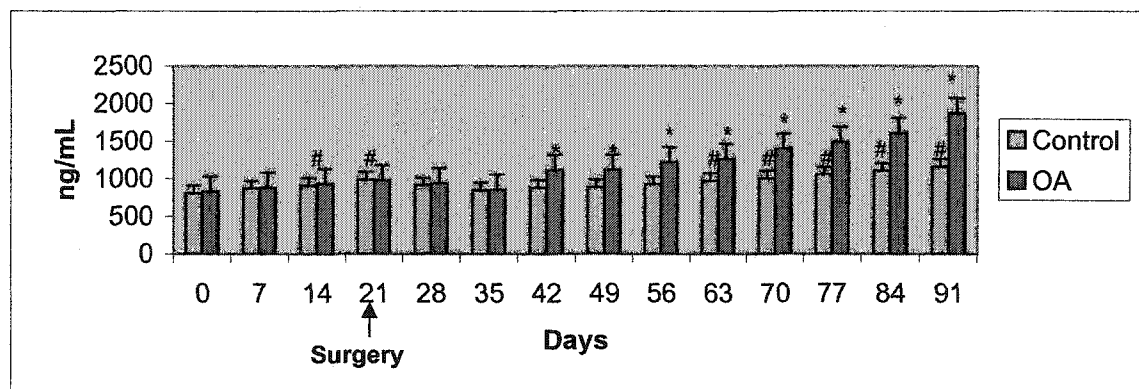


Figure 6. The mean ($\pm$ SEM) concentration of CPII in serum. The levels of CPII slightly increased with exercise. On days 42-91, the levels of CPII was significantly higher in the OA group compared to the control group. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of CPII compared to day 0 in the control group ($P < 0.05$).

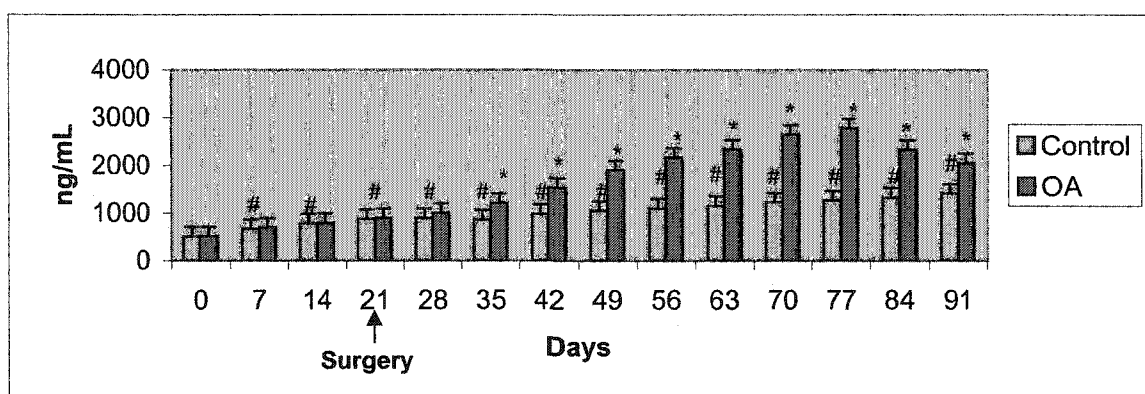


Figure 7. The mean ($\pm$ SEM) concentration of CS846 in serum. Starting on day 35, the levels of CS846 were significantly higher in the OA group compared to the control group. The significant differences continued until the end of the study. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of CS846 compared to day 0 in the control group ($P < 0.05$).

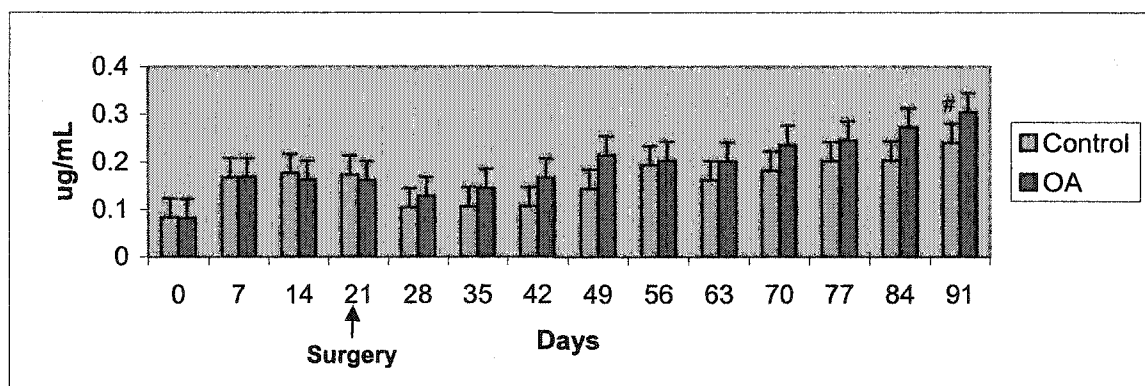


Figure 8. The mean ($\pm$ SEM) concentration of 234CEQ in serum. The levels of 234CEQ increased with exercise and then decreased during the resting weeks (weeks 4 and 5). One week after resuming the exercise (day 42), the levels of 234CEQ started to increase and continued increasing until the end of the study (day 91). Starting on day 28, the levels of 234CEQ in the OA group were higher than those in the control group. However, there was no significant difference between the two groups. The # indicates that day 91 is the only day that significantly has higher levels of 234CEQ compared to day 0 in the control group ($P < 0.05$).

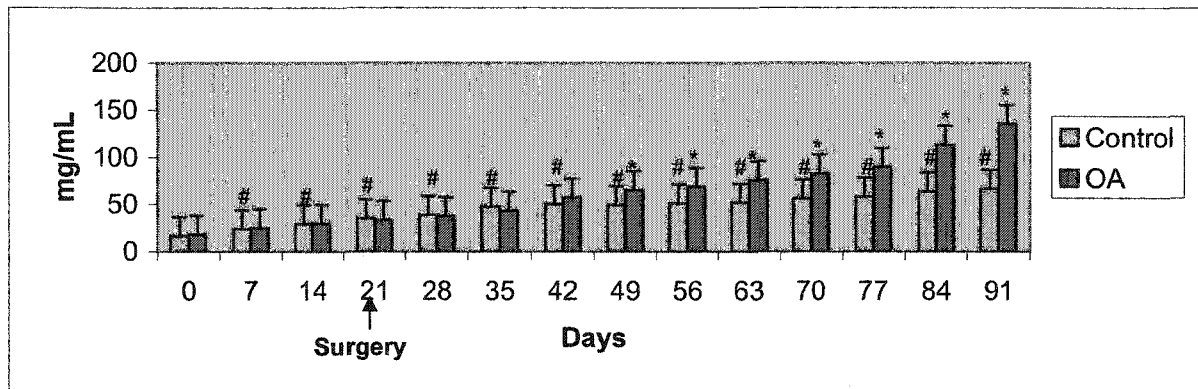


Figure 9. The mean ($\pm$ SEM) concentration of sGAG in serum. Starting on day 49, the levels of sGAG were significantly higher in the OA group compared to the control group. The significant differences continued until the end of the study. The * indicates the significantly elevated levels in the OA group compared with the control group ($P < 0.05$). The # indicates the days that significantly have higher levels of sGAG compared to day 0 in the control group ($P < 0.05$).

Table 2. This table shows values of R^2 between the synovial fluid (SF) and serum (S) biomarkers among the groups [OA, OA-normal (OA-N) and control (C)]. The * indicates significance at $P < 0.05$.

Biomarker	SFOA vs SOA	SFOA-N vs SOA	SFC vs SC
Osteocalcin	0.14	0.1	0.06
COL2-3/4C _{short}	0.81*	0.56*	0.5*
COL1-3/4C _{short}	0.52*	0.22*	0.27*
CPII	0.53*	0.48*	0.15*
234CEQ	0.18*	0.18*	0.01
CS846	0.44*	0.22*	0.5*
sGAG	0.79*	0.67*	0.69*

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II. CrossLapsTM one step ELISA, Osteometer BioTech A/S, Herlev, Denmark

III. ELISA Amplification System, GIBCO BRL, Grand Island, NY, USA

IV. Collagen II pro-peptide ELISA, HDM diagnostic & imaging inc, Toronto, Canada

V. CS846 ELISA assay, HDM diagnostic & imaging inc, Toronto, Canada

4 CORRELATIONS BETWEEN CLINICAL EXAMINATIONS, HISTOPATHOLOGICAL FINDINGS, AND BIOMARKER CONCENTRATIONS OF EXERCISED AND OSTEOARTHRITIC HORSES

4.1 The Introduction

A synovial joint contains the articulating surfaces of at least two bones covered with a thin layer of hyaline cartilage, soft tissues, and synovial fluid in the joint cavity. Hyaline cartilage is composed of an extracellular matrix containing mainly type II collagen and aggrecan.

Musculoskeletal diseases of the equine athlete are a major clinical problem with consequences ranging from lost training time to catastrophic injury and loss of life (Mohammed et al, 1991; Johnson, 1993; Wilson et al, 1993). Osteoarthritis (OA) is a common problem that affects horses and causes gross and histopathological changes in joint structures (Genovese, 1983; Rossdale et al, 1985; Todhunter and Lust, 1990; McIlwraith, 1996). Exercise might produce changes in the structural components of the joint similar to those seen in early OA. However, these changes are considered adaptive and not pathological changes.

The synovial membrane and articular cartilage often show gross and histological changes as a result of exercise and OA (Lust et al, 1981; Foland et al, 1994; Frisbie et al, 1997, 1998, and 1999a). The evaluations of gross and histopathological changes are valuable methods to detect changes occurring as a result of exercise and OA in the synovium and articular cartilage. However, several disadvantages exist when using these methods. In live horses, arthroscopic surgery is a method used to evaluate the joint grossly and to take biopsies from the affected synovium and cartilage. Therefore, this method is relatively expensive to apply. Moreover, the effect of individual variations due to sampling location that is seen when using this method will affect the precision of this method. A sample that is collected from the joint will reflect the changes in specific areas and may not reflect the general changes occurring in the joint. This method might not be a good method to detect early changes of OA. In fact, several early changes in joint structure might occur before the appearance of gross and histopathological changes. Earlier diagnosis of OA would be beneficial and potentially alter the course of disease through earlier interventions.

Several methods can be used to diagnose OA in equine joints. The major differences among these methods are how early in the disease process they can detect OA, and how strong they correlate with the gross and histological changes in joint structures in early OA.

Clinical examination is a routine method used to evaluate lameness, which is the most common cause for early retirement of equine athletes

(Jeffcott et al, 1982). Both exercise and OA can cause lameness in horses (Todhunter et al, 1993, Kawcak et al, 2000). Although the clinical examination is a routine method used to detect lameness in the musculoskeletal diseases of horses, it cannot detect OA in its early stages. Therefore, new diagnostic methods are required to diagnose OA in its early stages.

Recent technology used to evaluate OA is the measuring of the levels of biomarkers in biological fluids. In normal situations, there is balance between synthesis and degradation of extracellular matrix components required to provide articular cartilage, synovium and subchondral bone with their biomechanical and functional properties. With exercise and OA however, this balance is abnormally altered and the degree of aggrecan and collagen turnover increased (Eyre et al, 1991; Jackson et al, 1998; Wallace, 2000).

With exercise, changes in the articular cartilage matrix might be limited and considered an adaptive method that the horse utilizes to increase its ability to withstand increased loading. With OA however, the degree of changes seems to be beyond normal adaptation. Once released to the matrix, breakdown and synthesis products enter the synovial fluid, blood, and urine (Levick, 1992). The concentrations of some of these metabolites can be assessed in the biological fluids and used as specific biomarkers for OA. Several biomarkers of cartilage and synovium metabolism have been evaluated in patients with OA.

Inflammation of synovial membrane (synovitis) occurs commonly with OA in horses (McIlwraith and Van Sickle, 1981; McIlwraith, 1982). Joint effusion is the clinical sign of synovitis. Histologically, an inflamed synovial membrane can be identified by cellular infiltration, synovial intimal hyperplasia, subintimal edema, fibrosis, and increased vascularity (Foland et al, 1994). When synovitis occurs, degradative enzymes and their mediators are released from the synoviocytes (Martel- Pelletier and Pelletier, 1987; Trumble et al, 2001). Some of these mediators can be assessed and used as markers of synovial membrane inflammation. Of these mediators, prostaglandin E₂ (PGE₂) is a marker that can be assessed in synovial fluid. The concentration of PGE₂ has been measured in body fluids from exercised and osteoarthritic joints (Watanabe et al, 1991; Mitten et al, 1995; May et al, 1994; Gibson et al 1996; Kawcak et al, 1997).

The concentrations of the C-terminal propeptides of type II procollagen (CPII) and chondroitin sulfate epitope 846 (CS846) have been used as biomarkers of type II collagen and aggrecan synthesis, respectively. The fluid levels of these biomarkers have been reported to be higher in humans and horses with osteochondral fragmentation (OCF) than in normal controls (Frisbie et al, 1999b, Lohmandar et l, 1996 and 1999).

Several biomarkers of collagen degradation have been developed and one of these assays detects the COL2-3/4C_{short} neoepitope present on type I and II collagen fragments generated by collagenase cleavage (Billinghurst et al, 1997). In an *in vitro* study, it has been found that the levels of COL2-

3/4C_{short} in articular cartilage from osteoarthritic humans were significantly higher compared to levels in adult nonarthritic cartilage (Billinghurst et al 1997). In a recent study, a new antibody that specifically recognizes a neoepitope on collagenase-cleaved types II collagen in equine cartilage, the 234CEQ neoepitope, was developed (Billinghurst et al, 2001). It has been reported that cartilage from an osteoarthritic joint of a horse had increased staining for the 234CEQ neoepitope compared to nonarthritic cartilage (Billinghurst et al, 2001).

Several degradation biomarkers of articular cartilage aggrecan have been evaluated in biological fluids. Sulfated glycosaminoglycan (sGAG) is the most common biomarker that can be used as a biomarker of aggrecan turnover. Studies have shown that both exercise (Brama, 1999; Murray et al, 2001) and OA (Little et al, 1990; Alwan et al, 1991) stimulated increased levels of sGAG in the biological fluids of horses.

The purpose of this study was to evaluate clinical examination, gross changes, and histopathological changes in the joints of exercised horses with and without osteochondral fragmentation and correlate these with synovial fluid and serum biomarker concentrations. Since the results of the biomarker analyses were explained in detail in the last two chapters, we will not repeat these here, but I will report on their correlations with the findings of the clinical examinations and the documented pathological changes.

4.2 Materials and Methods

4.2.1 Animals

Sixteen healthy 2-year-old horses were used in this study. Eleven were females and five were males. During a 2-week acclimatization period, all horses were dewormed and vaccinated.

The horses were randomly assigned to either the fragmented or the control groups. The fragmented horses were subdivided into two groups: the fragmented joints (OA) and the contralateral joints (OA-normal).

Therefore, there were a total of three groups: the OCF joints in the fragmented horses or OA group (n = 8 joints), the non-fragmented joints in the fragmented horses or OA-normal group (n = 8 joints), and the control group (n = 16). A sham arthroscopic surgery was performed on the horses belonging to the control group.

4.2.2 Osteochondral fragmentation model

In order to determine the effects of surgery itself on the results, arthroscopic surgery was performed on both intercarpal joints of each horse. Starting 8 hours before surgery, each horse was medicated with procaine penicillin (22,000 IU/kg bw IM for 3 days) and phenylbutazone (4.4 mg/kg bw *per os* for 3 days). Each horse was sedated with xylazine, anesthetized with ketamine and diazepam, and anesthesia was maintained with halothane in 100% oxygen through a semi-closed breathing system. Each horse was positioned in dorsal recumbency and the carpal joints were flexed 90°. After sterile preparation and synovial fluid aspiration, bilateral carpal

arthroscopic surgery was performed in a routine manner (McIlwraith, 1990). Two portals (each approximately 6 mm) were made in each joint, laterally and medially. The lateral portal was placed between the common digital extensor tendon and the extensor carpi radialis tendon and it was used for inserting the arthroscope into the joint. The medial portal was placed medial to the extensor carpi radialis tendon and it was used for inserting the instruments. This portal allowed an 8-mm curved osteotome to be directed perpendicular to the articular cartilage surface of the radial carpal bone at the level of the medial synovial plica. After complete arthroscopic examination of the entire joint, the osteotome was used to create an osteochondral fragment in the dorsal aspect of the distal radiocarpal bone. The fragment was allowed to remain adherent to the joint capsule proximally. A motorized arthroburr was used to debride the exposed subchondral bone between the fragment and parent bone (Frisbie et al, 1997 and 1998). A 15 mm wide defect bed for the 8 mm wide fragment was created and the debris was not actively flushed from the joint. A 2-0 nylon suture was used to close the arthroscopic portals using simple interrupted or cruciate patterns. The limbs were bandaged and the horses allowed to recover. Each horse was kept in a 3.65 x 3.65 m stall. Bandages were changed every 3-5 days and maintained for two weeks. Sutures were removed 10 days postsurgery.

4.2.3 Experimental design

Figure 1 summarizes the experimental design used in this study. On day 0, before beginning the exercise program, synovial fluid samples were obtained from each horse. Exercise consisted of running on a high-speed treadmill 5 days/week; trotting for two minutes (16-19 km/h), galloping for two minutes (32 km/h), and trotting for two additional minutes (16-19 km/h). On day 21, arthroscopic surgery was performed and the horses were rested for two weeks after surgery. The exercise started again on day 35 and continued until the end of the study (day 91). Synovial fluids were aseptically aspirated once a week from both intercarpal joints of each horse beginning on day 0 and until the end of the study (day 91). Due to a technical error, the collection of synovial fluid was missed on weeks 7 (day 49), 9 (day 63), and 11 (day 77) for all horses. From each joint, 4 ml of synovial fluid was collected and approximately 0.5 ml of this fluid was placed into a tube containing ethylenediaminetetraacetic acid (EDTA) for performing the conventional analysis of synovial fluid. The conventional analysis of synovial fluid included assessment of color, clarity, total protein, white blood cell count, differential cytology of white cells, and mucin clot formation. The remainder of the synovial fluid sample (3.5 ml) was placed into plain glass tubes, centrifuged at 3,000g for 15 minutes, and the supernatant was aliquotted in tubes and stored at -80°C until biochemical marker analysis.

4.2.4 Lameness examinations

Lameness examinations were performed at the beginning (days 0) and end (day 91) of the study on all horses. Lameness was monitored during a jog and graded 0 –5 for severity according to guidelines established by the American Association of Equine Practitioners (1991). Briefly, grade 0 = soundness at a jog; grade 1 = lameness that was difficult to observe and not consistently apparent regardless of circumstances (e.g. weight-carrying, circling, incline, and hard surface); grade 2 = lameness that was difficult to observe at a walk or during trotting in a straight line but consistently apparent under certain circumstances such as lounging; grade 3 = lameness that was consistently observable at a trot under all circumstances, grade 4 = lameness that was obvious at a trot; and grade 5 = minimal weight bearing while the horse was in motion. Carpal flexion for 45 seconds was also performed and the response at the trot was graded on a scale of 0-4 where 0 = no response, 1 = slight response, 2 = mild response, 3 = moderate response, and 4 = severe response. Similarly synovial fluid effusion was also graded 0-4 where 0 = normal; 1 = slight, 2 = mild, 3 = moderate, and 4 = severe.

4.2.5 Biomarker Assays

4.2.5.1 Articular cartilage formation and resorption markers

As explained in the second and third chapters of this dissertation.

4.2.5.2 Inflammatory marker

4.2.5.2.1 PGE₂

As explained in the second chapter of this dissertation.

4.2.6 Post mortem examination

After euthanasia, the mid carpal joints were dissected and the gross lesions were recorded. The gross lesions were evaluated for total articular cartilage erosion score (TE), full-thickness articular cartilage erosion score (FTE), partial-thickness articular cartilage erosion score (PTE), and hemorrhage score (HM). Each variable was graded from 0 to 4 where 0 = normal; 1 = slight changes; 2 = mild changes; 3 = moderate changes; and 4 = severe changes. In addition, kissing lesions (KL) and soft tissue adhesion (SFA) scores were graded 1 = no lesion or 2 = lesions present.

4.2.7 Histologic evaluation of synovial membrane

Dorsal to the distal radiocarpal bone, synovial membrane samples were harvested from each intercarpal joint and fixed in 10% buffered formalin for hematoxylin and eosin staining (H&E). The sections were evaluated by three investigators and blindly graded for cellular infiltration, synovial intimal hyperplasia, subintimal edema, subintimal fibrosis, and vascularity. The graded scale for each variable was 0-4 where 0 = normal; 1 = slight changes; 2 = mild changes; 3 = moderate changes; and 4 = severe changes.

4.2.8 Histologic and histochemical evaluation of articular cartilage

Approximately 5 mm² pieces of articular cartilage pieces were harvested from the radial carpal, third carpal (radial facet), and fourth

carpal bones. These pieces were placed in 10% buffered formalin for seven days. From each piece of articular cartilage, two sections were prepared. One section was stained with H&E, and the other was stained with safranin O fast green (SOFG).

Articular cartilage sections stained with H&E were blindly evaluated by a pathologist for articular cartilage fibrillation, chondrocyte necrosis, chondrone formation, and focal cell loss (Frisbie et al, 1998). Articular cartilage fibrillation was graded on a scale from 0 to 4 where 0 = no fibrillation; 1 = surface fibrillation; 2 = clefts into the tangential zone; 3 = clefts into the radiate zone; and 4 = clefts into the calcified cartilage layer (Frisbie et al, 1998). Chondrocyte necrosis, chondrone formation, and focal cell loss were graded on a scale of 0 to 4 where 0 = normal; 1 = slight changes; 2 = mild changes; 3 = moderate changes; and 4 = severe changes (Frisbie, 1998).

Articular cartilage sections stained with SOFG were blindly evaluated by a pathologist for intensity of staining in the tangential, intermediate, radiate territorial and radiate interterritorial zones. The grading scale used was 0 to 4 where 0 = no stain uptake; 1 = slight stain uptake; 2 = mild stain uptake; 3 = moderate stain uptake; and 4 = normal stain uptake (Frisbie, 1998).

4.2.9 Statistical Analysis

Data normality was checked using plots of residual versus predicted values. If normality assumption was not met, the data was transformed

using natural logarithm (log) and normality reverified. In cases where log transformations were required, statistics analysis was performed on the transformed data.

Both the effects of exercise and group have been determined in this study. A mixed model analysis of variance (ANOVA) was used to analyse the dependent variables that were measured at two or less time points. Independent variables in this model were day, group, joint, and the interaction between these variables. When individual comparisons were made, a Least Square Means was utilized. Pearson correlation coefficients were calculated between the levels of biomarkers and scores of lameness, flexion, gross lesion, and histopathological changes. Values of the correlation coefficients were classified into four classes: slight ($r \leq 0.25$), mild ($0.25 < r \leq 0.50$), moderate ($0.50 < r \leq 0.75$), and strong ($0.75 < r \leq 1$) correlations. The significance level was set at $P < 0.05$.

4.3 Results

4.3.1 Animals

On day 0, all horses were healthy with no significant carpal lameness at a jog, they were unresponsive to carpal flexion tests, and there were no palpable carpal effusions. Within the OA group, there were no significant differences between the left and right OCF.

4.3.2 Experimental design

No prior lesions were noted in any joints during arthroscopic surgery except in one horse, which had partial tearing in the medial palmar intercarpal ligament in both intercarpal joints. Since no clinical signs were observed due to this lesion, this horse was not excluded from the study. For all horses, recovery from anesthesia was smooth and none required additional analgesia beyond three days of phenylbutazone administration.

4.3.3 Lameness examinations

The lameness and flexion scores for the three groups are shown in table 1. On day 91, the lameness scores in the OA group were significantly higher than those in the OA-normal and control groups and compared to base line scores ($P < 0.001$) (Figures 2). There was no significant difference in the lameness scores between the OA-normal and control groups between day 0 and day 91.

On day 91, the flexion scores in the OA group were significantly higher than those in the OA-normal and control groups ($P < 0.001$). Furthermore within each group, the flexion scores were higher on day 91 compared to the baseline on day 0. This increase was statistically significant in the OA groups ($P < 0.001$) and also in the other two groups ($P < 0.05$) (Figures 3). Table 2 shows the correlations between the lameness and flexion scores and the levels of serum biomarkers. The correlations between the lameness and flexion scores and the levels of biomarkers of aggrecan (CS846) and collagen (CPII) synthesis were higher than those with the levels of

biomarkers of aggrecan (sGAG) and collagen (COL2-3/4C_{shorts} 234CEQ) degradation. No significant correlations were found between the lameness and flexion scores and the biomarker levels in synovial fluid.

In this study, we found that the group ($P < 0.01$), time ($P < 0.001$), and the interaction of group and time ($P < 0.001$) significantly affected the effusion scores starting one week postsurgery, and continued until the end of the study (Figure 4). Over time, the effusion score increased in the control group in the weeks post-surgery compared to those before surgery and reached its highest levels in the first three weeks post surgery. Starting on day 56, the effusion scores slightly decreased and continued decreasing until the end of the study. Starting one week post-surgery (day 28) and until the end of the study, the effusion scores for the OA group were significantly higher than those in the OA-normal and control groups (Figure 4). On days 84 and 91, the effusion scores for the OA-normal group were also significantly higher than those in the control groups (Figure 4). The effusion scores were significantly correlated with the synovial fluid biomarkers including CS846 ($r^2 = 0.26$), sGAG ($r^2 = 0.25$), PGE₂ ($r^2 = 0.41$), CPII ($r^2 = 0.18$), COL2-3/4C_{short} ($r^2 = 0.16$) and 234CEQ ($r^2 = 0.10$).

4.3.4 Post mortem examination

All fragments were firmly readhered to the underlying parent bone. A thick discolored (reddish purple) synovium was attached to each fragment. All third carpal bones in the joints containing fragments had at least partial thickness cartilage erosions. Figures 5 shows photomicrographs of the third

carpal bones from OA, OA-normal and control groups. It can be seen that full-thickness articular cartilage erosions in OA group were more extensive than in the other two groups. Moreover, the full-thickness articular cartilage erosions in the OA-normal group were more extensive than those in the control group. Figure 6 shows the mean gross variable scores in the three groups. The mean scores of total erosion (TE), hemorrhage (HM), full thickness erosion (FTE), kissing lesions (KL), and soft tissue adhesion (SFA) were significantly higher in the OA group compared to the other two groups ($p < 0.01$). There was no significant difference among the three groups in the scores for partial thickness erosion (PTE). Furthermore, the scores of FTE in the OA-normal group were significantly higher than those in the control group ($P < 0.05$). Positive correlations were found between the scores of TE and HM ($r^2 = 0.30$), TE and SFA ($r^2 = 0.37$), and TE and KL ($r^2 = 0.27$). Furthermore, there were also positive correlations between HM and SFA ($r^2 = 0.50$), KL and SFA ($r^2 = 0.49$), and between HM and FTE ($r^2 = 0.31$).

When analyzing the relationship between the levels of biomarkers and the necropsy variables, strong positive correlations were noted between the levels of synovial fluid and serum sGAG and the TE ($r = 0.79$ and 0.77 , respectively). Strong positive correlations were also noted between the levels of synovial fluid CS846 and CPII and TE ($r^2 = 0.52$ and 0.49 , respectively). Moderate positive correlations were found between serum CS846 and CPII and TE ($r^2 = 0.43$ and 0.32 , respectively). In addition, moderate positive correlations were also found between synovial fluid and serum COL2-

$3/4C_{\text{short}}$ and TE ($r^2 = 0.36$ and 0.44 , respectively). The KL scores were positively correlated with synovial fluid and serum 234CEQ ($r^2 = 0.41$ and 0.39 , respectively), and COL234C_{short} ($r^2 = 0.39$ and 0.22 , respectively).

4.3.5 Histology of synovial membranes

Figure 7 shows a photomicrograph from synovial membrane stained with H & E obtained from the three groups. Figure 8 shows the mean score of each synovium histopathological variable in the three groups. The scores of cellular infiltration (CINF) and subintimal vascularity (VASC) were significantly higher in the OA and OA-normal groups compared to the control group ($P < 0.01$). There were no significant difference in the scores for intimal hyperplasia (INHP), subintimal edema (SIED) and subintimal fibrosis (SIFS) among the three groups. No significant correlations were found between the serum or synovial fluid markers and any of the synovial membrane histopathology scores.

4.3.6 Histologic evaluation of articular cartilage

Figure 9 shows a photomicrograph from a 5um section of articular cartilage stained with H&E stain obtained from the three groups. The presence or absence of the fragment and its location had an effect on the scores for fibrillation, chondrone formation, chondrocyte necrosis, and focal cellular loss. Table 3 shows the comparisons among the groups in the scores for the articular cartilage histopathologic variables obtained from the radial, third, and fourth carpal bones.

The scores of cellular fibrillation in the OA and OA-normal groups were significantly higher than those in the control group ($P < 0.05$). Moreover, the scores of chondrone formation and focal cell loss ($P < 0.05$) were significantly higher in the articular cartilage sections obtained from the radiocarpal bones from the OA group compared to those from the other two groups (Figure 10). There were no significant differences among groups in the scores for chondrocyte necrosis although they were slightly higher in the OA group compared to the other two groups.

Figure 11 shows the scores for all histopathologic variables in the articular cartilage tissue sections obtained from the third carpal bones. The scores of all variables were significantly higher in the OA group compared to the other two groups ($P < 0.01$). In addition, the scores of fibrillation were significantly higher in the OA-normal group compared to the control group ($P < 0.05$).

Figure 12 shows the scores for histopathologic variables in the articular cartilage tissue sections obtained from the fourth carpal bones. There were no significant differences among the groups for the scores of all the variables.

A positive correlation was found between chondrone formation and fibrillation ($r^2 = 0.62$) and between chondrone formation and chondrocyte necrosis ($r^2 = 0.51$). Furthermore, there was a positive correlation between chondrocyte necrosis and fibrillation ($r^2 = 0.40$).

Tables 4 and 5 show the correlations between the scores for the articular cartilage histopathologic variables and the levels of biomarkers in synovial fluid and serum, respectively. In addition, there were significant positive correlations between fibrillation scores and the following variables: total protein ($r^2 = 0.43$), neutrophil percentage ($r^2 = 0.53$), and the effusion scores ($r^2 = 0.43$).

4.3.7 Histochemical evaluation of articular cartilage

Figure 13 shows a photomicrograph of a 5 μ m section of articular cartilage stained with SOFG obtained from the third carpal bones for each group. Figure 14 shows that the mean total score for SOFG staining in the OA group was significantly lower than the mean scores in the other two groups ($P < 0.05$).

The comparisons among groups in the scores of SOFG staining intensity in the radial, third and fourth carpal bones are shown in Table 6. Figure 15 shows the staining intensity scores in the different zones in the articular cartilage tissues obtained from the radiocarpal bones and stained by SOFG. The scores of staining intensity in the tangential, intermediate and radiate interterritorial zones were significantly lower in the OA group compared to the other two groups ($P < 0.05$). No significant differences were found between the groups in the scores of staining intensity of the radiate territorial zone.

The staining intensity scores in the different zones of the articular cartilages obtained from the third carpal bones and stained by SOFG are

shown in Figure 16. The staining intensity scores in the tangential, intermediate and radiate interterritorial zones were significantly lower in the OA group compared to the other two groups ($P < 0.05$). In addition, the staining intensity scores in the tangential, and radiate interterritorial zones were significantly lower in the OA-normal group compared to the control group ($P < 0.05$). There were no significant differences between the groups in the scores of staining intensity of the radiate territorial zone.

In the articular cartilage obtained from the fourth carpal bones and stained with SOFG, there were no significant differences among the groups in the staining intensity scores for all the zones (Figure 17).

Strong positive correlations were found between the SOFG scores for the radiate territorial and the radiate interterritorial ($r^2 = 0.59$) and the radiate interterritorial and the tangential zones ($r^2 = 0.46$). In addition, there was moderate positive correlation between the SOFG scores for the tangential and the intermediate zones ($r = 0.36$).

Table 7 shows negative correlations between the staining intensity score in each articular cartilage zone and the synovial fluid biomarkers. Significant negative correlations were found between the staining intensity of all articular cartilage zones and the levels of sGAG in synovial fluid samples. No significant correlations were found between the staining intensity score in each articular cartilage zone and the serum biomarker levels.

4.4 Discussion

It is believed that the osteochondral fragmentation (OCF) model used in this study has the ability to produce adequate pathological changes to provide an acceptable equine model of traumatic osteoarthritis (Frisbie et al, 1997; Kawcak et al, 1997).

In this study, we found that the continuation of exercise in horses with experimentally induced osteochondral fragmentation of the distal radial carpal bone resulted in an increased lameness response to carpal joint flexion. It is important to note that exercise significantly increased the scores for carpal flexion response in healthy exercised horses compared to scores obtained before the exercise regimen, but no significant effect of exercise on lameness was seen for these same horses. This suggests that the carpal flexion test is very sensitive in detecting exercised-induced changes in the intercarpal joint and therefore, we would recommend that it be performed with the lameness examination.

Osteochondral fragmentation is common in young racehorses and the distal dorsal aspect of the radial carpal bone is the most common site for this lesion (McIlwraith et al, 1987). It has been thought that the extreme hyperextension of the immature carpus during racing and training is the major cause of osteochondral fragmentation (Bramlage et al, 1988). Several studies have evaluated the effects of exercise and/or osteochondral fragmentation on the clinical status of horses. Kawcak et al (2000) have reported that 4 out of 6 healthy exercised horses became lame approximately

three months after beginning exercise on a treadmill, and all were lame after three additional months. In another study using the same OCF model, Kawcak et al (1998) have found that all horses with osteochondral fragmentation became lame in the affected limbs 72 days postsurgery, and a lameness grade of 2/5 was the highest score. In our study, the highest lameness and flexion scores were 3/5 and 4/4, respectively. However, the length of the current study (91 days) was different from that of the Kawcak study (72 days).

Positive correlations were seen between the lameness and flexion scores and the serum biomarker levels evaluated in this study (Table 2). The serum levels of biomarkers indicating aggrecan turnover and type II collagen synthesis were significantly and positively related to the lameness and flexion scores (Table 2). In addition, the flexion test scores were related to serum values of the biomarker indicating type I and II collagen degradation (Table 2). We found no significant correlations between the lameness and flexion scores and the articular cartilage biomarkers in synovial fluid. The explanation for this might be because there was a whole body response to the single joint lameness in order to compensate for the loss of normal function of that joint. This might lead to increases in the metabolic activity of skeletal tissues that have lower turnover in the normal situation. That may explain the correlations between the lameness and flexion scores and the serum biomarkers and not with the synovial fluid levels of biomarkers from the affected joint.

Joint effusion is a clinical sign that can be used to detect joint inflammation. We found that the significant increase over time in the effusion scores within the control group started one week postsurgery and then decreased gradually until the end of the study. This indicates that exercise caused inflammation in the joint, but the degree of inflammation decreased over time. This suggests that a fixed level of exercise might produce a significant degree of inflammation in equine joints for a limited period of time and the inflammation will gradually reduce until reaching baseline levels. In nature, athletic horses encounter various levels of exercise. Therefore in order to study the effect of exercise on the joint tissues of horses, I suggest that the exercise regimen used in our model should be changed to make this model much closer to what happens with naturally exercised horses. Instead of exercising the horse five days/week at same level of exercise, I think decreasing the level of exercise in the second and fourth days might help getting more accurate response to the effect of exercise on the joints. This will allow the horse to face various levels of exercise which resembles what happens in nature.

The significant increase in effusion scores for the OA group compared to those in the other two groups started one week postsurgery and continued until the end of the study. This indicates that the OCF in the joint increased the joint inflammation. This might suggest that the effusion score is a very sensitive clinical tool that can be used in the diagnosis of joint inflammation. The moderate correlation between the levels of synovial fluid PGE_2 and the

effusion scores that we found in this study ($r^2 = 0.41$) supports this suggestion.

On days 84 and 91, the effusion scores of the OA-normal group were significantly higher than those in the control group. This suggests that the osteochondral fragmentation in one joint of a horse affects the health of the contralateral joint of the same horse. The explanation for this increase in effusion may be due to an increase use of the contralateral limb and its joints in an effort to minimize the use of the OCF joints. This would in turn lead to increase loading of the contralateral joints with increased inflammation.

Positive correlations were found between the synovial effusion scores and the fluid levels of the articular cartilage biomarkers assessed in this study. In particular, there were significant correlations between the synovial effusion scores and the biomarker levels of aggrecan turnover. The correlations between the synovial effusion scores and the biomarker levels of collagen turnover were relatively small. This suggests that aggrecan turnover like the appearance of synovial effusion is a reflection of acute inflammatory changes occurring in the model.

Upon gross examination, we found that the scores of all the gross variables (except the partial thickness erosion) were significantly higher in the OA group compared to the other two groups (Figure 6). The fact that the early signs of OA are associated with increased scores for these variables supports the contention that the model used in this study is a good model of naturally occurring OA in the horse. The average scores of total erosion (TE)

and full-thickness erosion (FTE) of cartilage were significantly higher in the OA group compared to the other two groups. Since articular cartilage degradation is the most characteristic feature seen in OA (McIlwraith, 1982), this suggests that training a horse that has osteochondral fragmentation of the distal dorsal aspect of the radiocarpal bone leads to cartilage changes consistent with those seen in OA. It is recommended that horses with chip fragments in this location not be raced or trained until the removal of the chips (McIlwraith, 1987), otherwise, articular cartilage damage will occur and that leads to OA (McIlwraith, 1987). Furthermore, we found that the scores of FTE in the OA-normal group were significantly higher than those in the control group. This suggests that the gross changes of the healthy joint were significantly affected with the occurrence of an OCF lesion in the contralateral joint of a horse. This finding supports the increased use of the contralateral joints suggested by the increased effusion scores in these joints.

The kissing lesions (KL) in the third carpal bones were the most common defects seen in the carpal bones. The kissing lesions are found in the surfaces that oppose the fragment in the joint. In the KL areas, there were articular cartilage erosions and pink discoloration beneath the articular cartilage, and these lesions might be gross evidence of collagen degradation in the articular cartilage. The significant correlations we found between the KL scores and the biomarker levels of collagen degradation support this result. We found that there was a significant difference in the KL scores between the OA group and the other two groups. Barr et al (1994) have

reported that kissing lesions in the third carpal bones were grossly more severe in exercised horses with osteochondral defects in radial and intermediate carpal bones than in control non-exercised joints.

Significant correlations were found between TE and HM, SFA, and KL. This is an expected result because the appearance of these variables (TE, HM, SFA, and KL) is considered a common early gross sign of OA (McIlwraith, 1996).

In this study, we found that there were significant positive correlations between the total erosion (TE) scores and the fluid levels of the articular cartilage biomarkers. These findings would be expected since the observed erosions occurred in articular cartilage. The significant correlations were between the TE and the synovial fluid and serum levels of the markers CS846, sGAG, CPII, and COL2-3/4C_{short}. This suggests that the rate of aggrecan and collagen turnover in the articular cartilage increased with the degree of articular cartilage erosion. As might be expected, the kissing lesion (KL) score was positively correlated with the levels of 234CEQ and COL2-3/4C_{short}. This suggests that there was increased collagen degradation with increase severity of these kissing lesions.

Other studies have evaluated the gross changes seen in the joints with OA in various species. In horses using the same model, Frisbie et al (1997 and 1998) have reported gross changes in articular cartilage and synovium similar to those seen in the current study. In dogs using the Pond-Nuki model, Brandt et al (1991) have found that there was full-thickness erosion

(FTE) of cartilage on at least one-third of the central region of the medial femoral condyle and on the lateral tibial plateau.

The histopathologic changes in articular cartilage sections showed that the scores of fibrillation, chondrone formation, chondrocyte necrosis, and focal cell loss for the OA group were significantly higher than those in the other two groups. This indicates that there was greater histological evidence of articular cartilage degradation in the OA group compared to that in the other two groups. This suggests that this model can lead to classic histological lesions of OA. In this study, we found that the articular cartilages of the radial and third carpal bones were the most degraded amongst the carpal bones examined in the fragmented equine intercarpal joint. Other studies, using a similar model, have reported that the articular cartilages of these two bones are the most commonly affected in the equine intercarpal joints (Frisbie et al, 1997, 1998 and 1999a). It has been reported that the distal radial carpal bone is the most common site for chip fractures in equine carpal joints associated with severe loss of its articular cartilage (McIlwraith et al, 1987a). Using the same model, Frisbie et al (1997, 1998, 1999a) have found that the third carpal bones were commonly the regions with the most articular cartilage degradation (Frisbie et al, 1997, 1998 and 1999a). It has been theorized that these bones are under high loads (Palmer et al, 1986), usually not completely mature in the young racing horse and under extreme hyperextension during exercise (Bramlage et al, 1988). In our study, a positive correlation was found between the scores of chondrone

formation and fibrillation, the scores of chondrone formation and cellular necrosis and between the scores of cellular necrosis and fibrillation. These correlations were expected since the histopathologic signs of OA in the articular cartilage include the appearance of fibrillation, chondrone formation, and necrosis.

Interestingly, we found that the fibrillation scores in articular cartilages from the radial and third carpal bones of the healthy contralateral joints in the fragmented horses were significantly higher than those from the control horses. This once again suggests that the osteoarthritic joints had effects on the healthy contralateral joints. We believe that the decreased use of the diseased joints led to increased loading on the healthy contralateral joints and that in turn led to the appearance of pathologic changes in the cartilages of these joints.

We found that the histopathological changes were related to the changes seen in the levels of serum and synovial fluid biomarkers. The synovial fluid and serum levels of aggrecan and type II collagen biomarkers were correlated with the histopathological scores for the articular cartilage. These correlations are evidence of the usefulness of measuring the body fluid levels of articular cartilage biomarkers as indicators for the degree of articular cartilage damage. From Tables 4 and 5, it can be seen that there are significant correlations between fibrillation and the biomarkers of both collagen and aggrecan metabolism. This suggests that the appearance of fibrillation is associated with increases in both collagen and aggrecan

turnover. The positive correlations that we found between the scores of fibrillation and the synovial fluid levels of PGE₂ suggested that fibrillated articular cartilage was associated with inflammation (synovitis) in this model. This finding is supported by the significant correlation that we found between the fibrillation scores and the scores of synovial fluid total protein and effusion scores. The positive correlation between the scores of fibrillation and the percentage of neutrophils in synovial fluid might suggest that these cells may play a role in the production and/or activation of the cartilage degradative enzymes. The reason that there were no significant correlations between the fibrillation scores and the serum levels of sGAG may be because more degradation might occur to the sGAG in the lymphatic system before it reaches the blood stream. In addition, articular cartilage fibrillation may be more associated with collagen and not aggrecan degradation. Therefore, it has more correlation with the collagen than the aggrecan biomarkers.

In this study, we found that the intensity of SOFG staining of articular cartilages obtained from the OA group were significantly lower than those from the other two groups (Figure 14). This suggests that there was greater aggrecan degradation and loss in the OA group compared to that other two groups. There are other studies that have evaluated the intensity of the SOFG stain in articular cartilage sections obtained from normal and arthritic joints. Frisbie et al (1997 and 1998), using the same OA model, have evaluated the effects of the intraarticular injections of corticosteroids in

equine joints with experimental OCF. They have reported that the staining intensity of the articular cartilage from the third carpal bone was the only area that showed significant differences between the treated and the non-treated joints (Frisbie et al, 1997 & 1998). Articular cartilage staining in the other carpal bones was not significantly different between the OCF and the control joints (Frisbie et al, 1997 & 1998).

We found in the current study that the scores of SOFG staining intensity in the third carpal bone were significantly lower in the OA-normal group compared to the control group. This again supports our conclusion that the affected joint has an effect on the healthy contralateral joint in the same horse. In our study, a positive correlation was found in the loss of staining intensity of the articular cartilage zones among each other. These findings suggest that the aggrecan loss occurred equally throughout the cartilage matrix in this model. In the radiate territorial zone, we could not find significant differences in the staining intensity scores between groups. This might be because the degradative enzymes had not been completely activated and/or because there was an increase in the amount of proteoglycan molecules released by the chondrocytes which masked the lost of these molecules. Since there was a significant negative correlation between the staining intensity scores in the territorial zone and the levels of synovial fluid sGAG, this suggest that high proteoglycan degradation might occur in this zone. This leads us to suggest that the increased synthesis of proteoglycan molecules might mask their significant degradation in the

radiate territorial zone and thus no significant differences in the staining intensity scores were found between groups. It has been suggested that the radiate territorial is a metabolically active zone that significantly contributes to the appearance of aggrecan fragments in body fluids (Kuettner and Thonar, 1998).

It has been reported that in adults with normal articular cartilage, the source of cartilage biomarkers appearing in body fluids is principally from the turnover of the components of the matrix (Kuettner and Thonar, 1998). In our study, we found there were negative correlations between the intensity of staining in the articular cartilage zones and the synovial fluid levels of biomarkers. The negative correlation was significant between the intensity of staining in the articular cartilage zones and the synovial fluid sGAG. This suggests that the source of sGAG in synovial fluid is likely coming from the degradation of aggrecan molecules in the articular cartilage matrix. Moke et al (1994) have reported that the degradation of newly synthesized molecules, which are released extracellularly, is a major source for the biomarkers appearing in body fluids. A weak negative correlation was found between the staining intensity of articular cartilage zones and the levels of CS846 in synovial fluid samples. This suggests that most of the newly synthesized aggrecan molecules remain in the matrix in normal cartilage and increasing amounts enter the synovial fluid with disease.

We found low negative correlations between type II collagen biomarkers and the staining intensity in the different zones of the articular

cartilage sections. This is expected because safranin-O staining of articular cartilage is considered a useful semiquantitative method to estimate proteoglycan content in the articular cartilage matrix and not collagen (Rosenberg, 1971). In order to evaluate the rate of collagen degradation histologically, articular cartilage sections should be stained with a special stain for collagen such as Sirius Red, and Mallory and Massons triple stains (Eurell and Van Sickle, 1998).

Grossly, the scores for hemorrhage (HM) and soft tissue adhesion (SFA) were significantly higher in the OA group compared to the other two groups. This suggests that there was greater soft tissue inflammation in the OA group compared to the other two groups. This was supported by the histopathological examinations. We found that the degrees of cellular infiltration and subintimal vascularity were significantly higher in the OA group compared to the control group. Inflammation of the synovial membrane (synovitis) is an important factor in stimulating the release and activation of proteolytic enzymes. It has been reported that synovitis and capsulitis are common in the joint as a result of repeated trauma (McIlwraith, 1982; Evans, 1992). McIlwraith and Van Sickle (1981) proved that synovitis could lead to osteoarthritis in horses. It has been reported that there was a correlation between synovial membrane inflammation, collagenolytic enzyme activity, cartilage breakdown and severity of disease (Nilsson and Olsson, 1973; Ehrlich et al, 1978; Pelletier et al, 1985 and 1994; Appel et al, 1988; Spiers et al, 1994). Matrix metalloproteinases (MMPs) are

important degradative enzymes whose levels in many tissues and fluids increase with inflammation (Martel-Pelletier et al, 1987). It has been shown that synovial cells (synoviocytes) are a rich source of proteolytic enzymes active against both collagen and proteoglycan (Spiers et al, 1994). Therefore, it is expected to find high levels of proteolytic enzymes resulting in increases in inflammation in the OCF joints compared to the control joints.

There are other studies that have found similar results. Frisbie et al (1997), using the same OCF model, described the effect of intra-articular corticosteroids therapy on joint tissues in horses. They found that the scores for inflammatory cell infiltration and subintimal fibrosis in the joints of exercised horses with experimentally induced OCF were significantly higher than those in the treated joints. In another study using the same model, Frisbie et al (1998) have reported that there were significant increases in synovial membrane vascularity and inflammatory cell infiltration in the OCF joints compared to the control joints. Similar results were found in dogs with anterior cruciate ligament transection (Brandt et al, 1991). Based on these results, it can be concluded that exercise with osteochondral fragmentation stimulates synovitis in equine joints.

Interestingly, we found that the degrees of cellular infiltration and subintimal vascularity were significantly higher in the OA-normal group compared to the control group. This again suggests that the fragmented joint in the same horse affected the healthy contralateral joint possibly because of the increased stress placed on the contralateral joint.

In summary, this study has showed that clinical examinations, and gross, histological and histochemical changes can be used to differentiate between the osteoarthritic and the adaptive (exercised) joints of horses. The clinical examinations, and gross, histological and histochemical changes were significantly higher in the osteoarthritic carpal joints than the exercised joints of horses.

The fluid levels of biomarkers were correlated with the changes seen in clinical examinations and gross, histological and histochemical changes. A positive correlation found between the levels of synovial fluid PGE₂ and the effusion score indicated that the effusion test is a very sensitive clinical tool that can be used in the diagnosis of synovitis in the carpal joint. Correlations were found between the gross, histological, and histochemical changes in articular cartilage and the synovial fluid and serum levels of the articular cartilage biomarkers. This suggests that the rate of aggrecan and collagen turnover in the articular cartilage increased with increase in articular cartilage pathology. This supports the usefulness of biomarkers in detecting OA in its early stages and differentiating the changes occurring in osteoarthritic joints from those occurring in healthy exercised joints.

Interestingly, this study showed that, over time, the osteoarthritic joint would negatively affect the healthy contralateral joint. This might occur as a result of increased use of the healthy contralateral joints in order to compensate and minimize use of the OCF joints by the horse.

4.5 Tables and figures

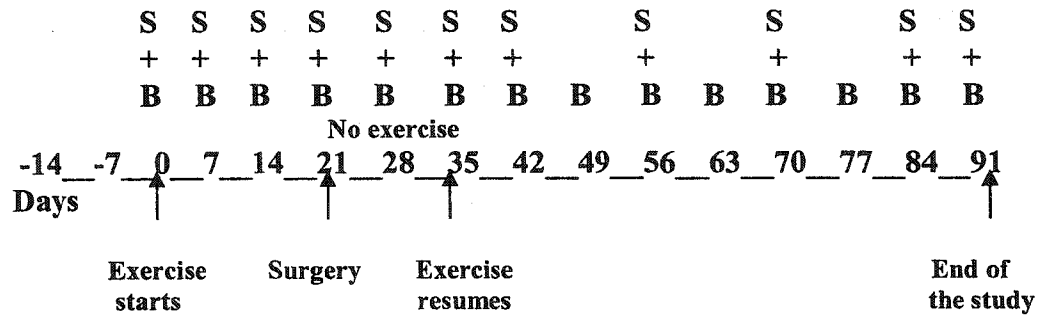


Figure 1. The timeline of the study is summarized in this figure. The study started two weeks before the beginning of exercise (-14) and finished on day 91. On day 21, surgery was performed on each horse. After that, the horses were rested for two weeks. Then, the horses resumed exercise and continued until the end of the study. Synovial fluids (S) were aseptically collected once a week from right and left mid carpal joints of each horse beginning on day 0 and until the end of the study (day 91) except on weeks 7 (day 49), 9 (day 63), and 11 (day 77), where the collection of synovial fluid was omitted for all horses. Blood samples (B) were also collected once a week from each horse through the jugular vein.

Table 1. The means ($\pm$ SD) of lameness and flexion scores for the three groups. The * indicates significant difference between day 91 compared to day 0. The # indicates significant difference between day 91 in OA group and the other two groups in the same day ($P < 0.05$).

Group	Lameness Score		Flexion Score	
	Day 0	Day 91	Day 0	Day 91
OA	0.14 ± 0.24	$2 \pm 0.81^* \#$	0 ± 0	$2.3 \pm 1^* \#$
OA-normal	0.14 ± 0.35	0.28 ± 0.48	0 ± 0	$0.63 \pm 0.42^*$
Control	0.14 ± 0.24	0.21 ± 0.38	0 ± 0	$0.70 \pm 0.53^*$

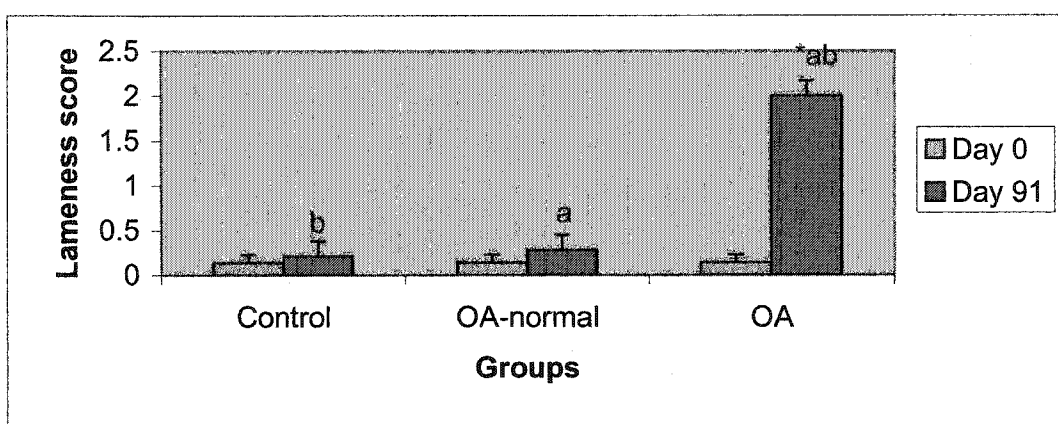


Figure 2. The mean lameness score ($\pm$ SEM) in the three groups. In the OA group, the lameness scores on day 91 were significantly higher than those on day 0. On day 91 the OA group had significantly higher scores than the other two groups in the same day. In the OA-normal and control groups, there were no significant differences between day 0 and day 91. The * indicates the significant scores on day 91 compared to day 0. The bars with same letters indicate significant differences between these bars ($P < 0.05$).

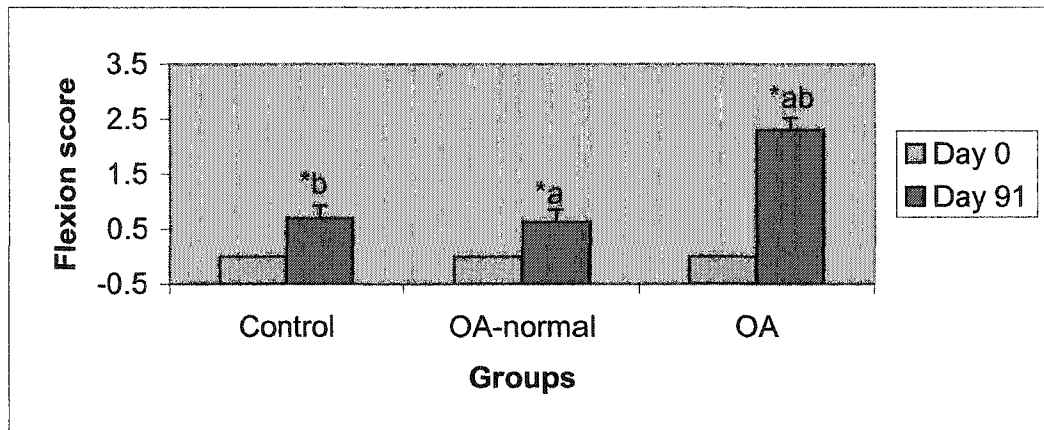


Figure 3. The mean carpal flexion scores ($\pm$ SEM) for the three groups. Within each group, the flexion scores on day 91 were significantly higher than those on day 0. The flexion scores on day 91 were significantly higher than those in the other two group. The * indicates the significant scores on day 91 compared to day 0. The bars with same letters indicate significant differences between these bars ($P < 0.05$).

Table 2. Values of R^2 between lameness and flexion scores and the serum levels of articular cartilage markers.

The values of R^2 between the flexion scores and the biomarker levels were higher than those between the lameness scores and the serum levels of articular cartilage markers.

The * indicates significance at $P < 0.05$.

Biomarker	Lameness score	Flexion score
GAG	0.37*	0.46*
CS846	0.39*	0.57*
COL2-3/4Cshort	0.01	0.39*
234CEQ	0.04	0.02
CPII	0.39*	0.52*

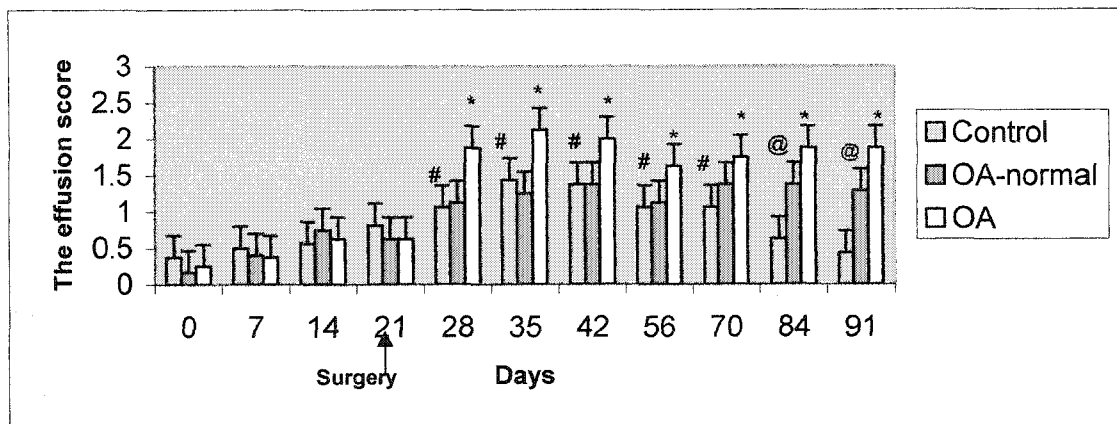


Figure 4. The weekly mean ($\pm$ SEM) effusion scores in the three groups. Starting on day 28-91, there was a significant increase in the effusion scores in the OA group compared to the other two groups (*). The @ indicates that OA-normal group significantly has higher scores compared to the control group. The # indicates the days that significantly have higher scores of effusion compared on day 0 in the control group ($P < 0.05$).

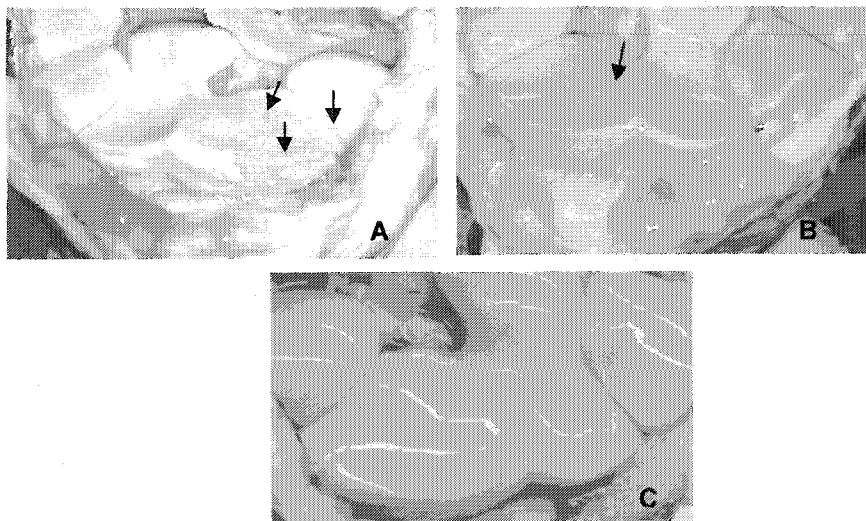


Figure 5. Photomicrographs of the 3rd carpal bones from OA (A), OA-normal (B) and the control (C) groups. Note more extensive full-thickness articular cartilage erosions in OA group compared to the other two groups. In addition, the OA-normal group has greater full-thickness articular cartilage erosions than the control group.

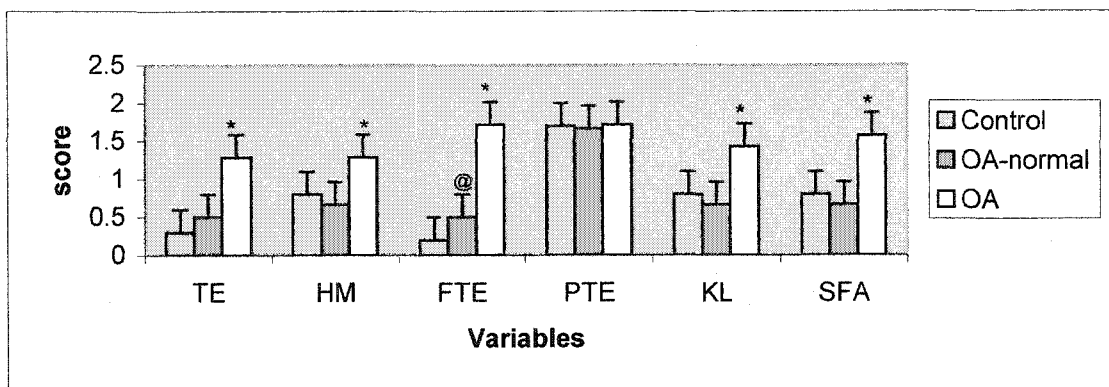


Figure 6. The mean ($\pm$ SEM) of gross variable scores in the three groups. The scores of total erosion (TE), hemorrhage (HM), full thickness erosion (FTE), kissing lesions (KL), and soft tissue adhesion (SFA) in the OA group were significantly higher than those in the other two groups (*). No significant difference was found among the groups in the scores for partial thickness erosion (PTE). The @ indicates that the scores of FTE were also significantly higher in the OA-normal group compared to the control group. $P < 0.05$.

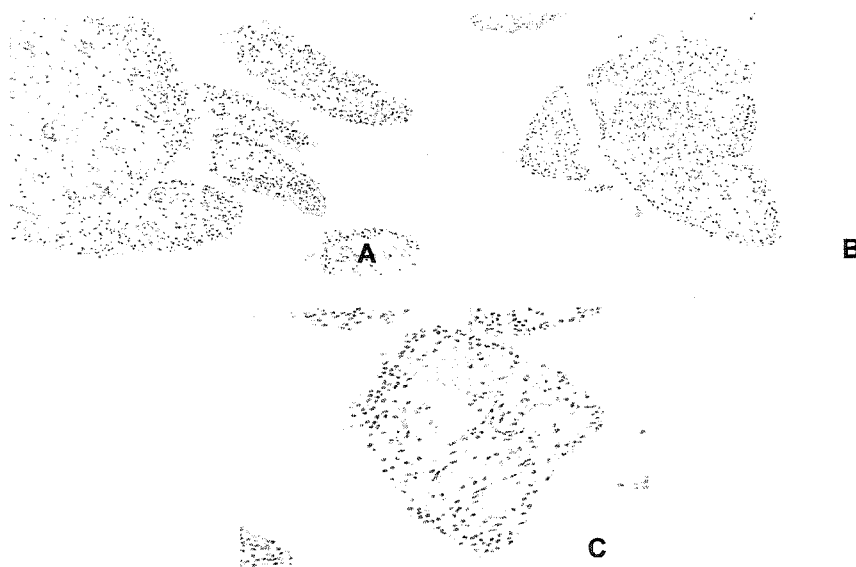


Figure 7. Photomicrographs of 5 μ m sections of synovial membrane obtained from the OA (A), OA-normal (B), and control (C) groups and stained with H&E. There is a significant increase in the scores of cell infiltration and vascularity in the OA and OA-normal groups compared to the control group.

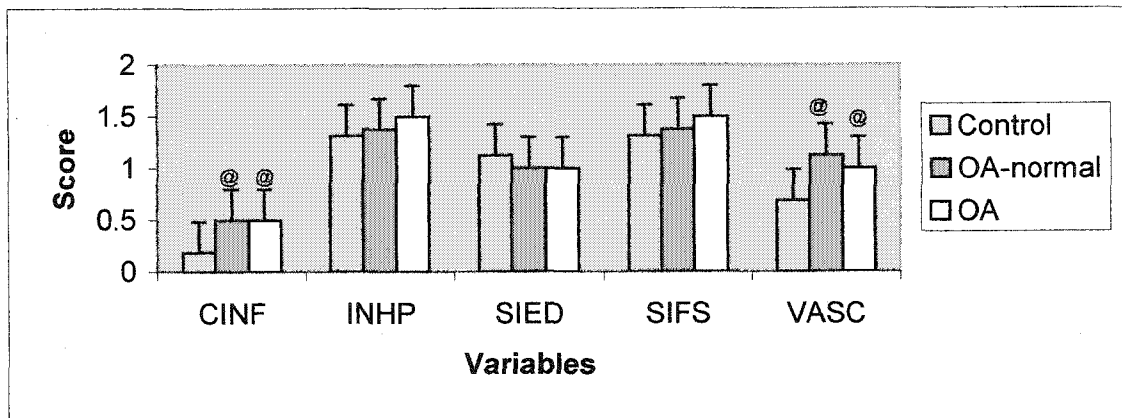


Figure 8. The means ($\pm$ SEM) of synovium histopathological variable scores in the three groups. The scores of cellular infiltration (CINF) and subintimal vascularity (VASC) were significantly higher in both the OA and the OA-normal groups compared to the control group (@). No significant differences were found among the groups in the scores of intimal hyperplasia (INHP), subintimal edema (SIED) and subintimal fibrosis (SIFS). $P < 0.05$

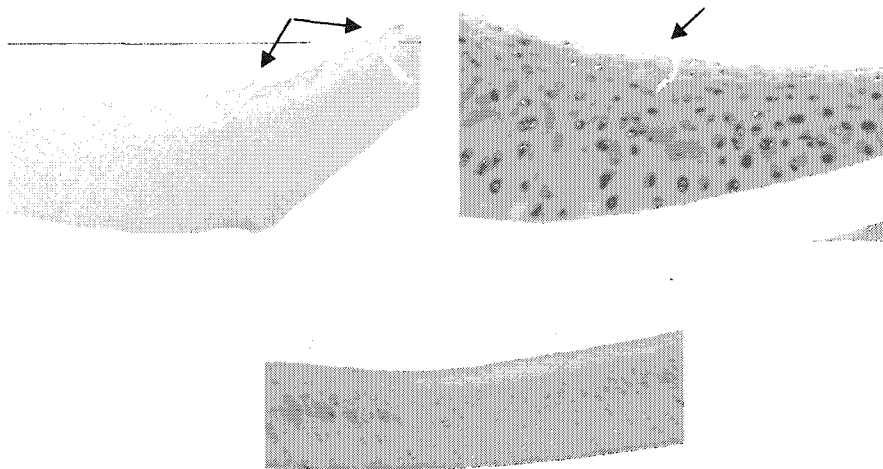


Figure 9. Photomicrographs of 5 μ m sections of articular cartilage obtained from the third carpal bone for the OA (A), OA-normal (B), and control (C) groups stained with H&E. Cellular fibrillation score is significantly higher in OA and OA-normal groups compared to the control group. The scores of chondrone formation and focal cell loss are higher in OA group compared to the other two groups.

Table 3. The comparisons between the groups in the scores of the histopathologic variables of the articular cartilage sections obtained from the radial, third, and fourth carpal bones and stained with H & E. The "Yes" indicates that the scores of the variable were significantly higher in the first group compared to the second group ($P < 0.5$). The "No" indicates no significant differences was found between the two groups in the scores of the variable at $P < 0.5$.

The bone	The comparisons	Fibrillation	Cell necrosis	Chondrone	Cell Loss
Radiocarpal bone	OA vs Control	Yes	No	Yes	Yes
	OA-normal vs Control	Yes	No	No	No
	OA vs OA-normal	No	No	Yes	Yes
Third carpal bone	OA vs Control	Yes	Yes	Yes	Yes
	OA-normal vs Control	Yes	No	No	No
	OA vs OA-normal	Yes	Yes	Yes	Yes
Fourth carpal bone	OA vs Control	No	No	No	No
	OA-normal vs Control	No	No	No	No
	OA vs OA-normal	No	No	No	No

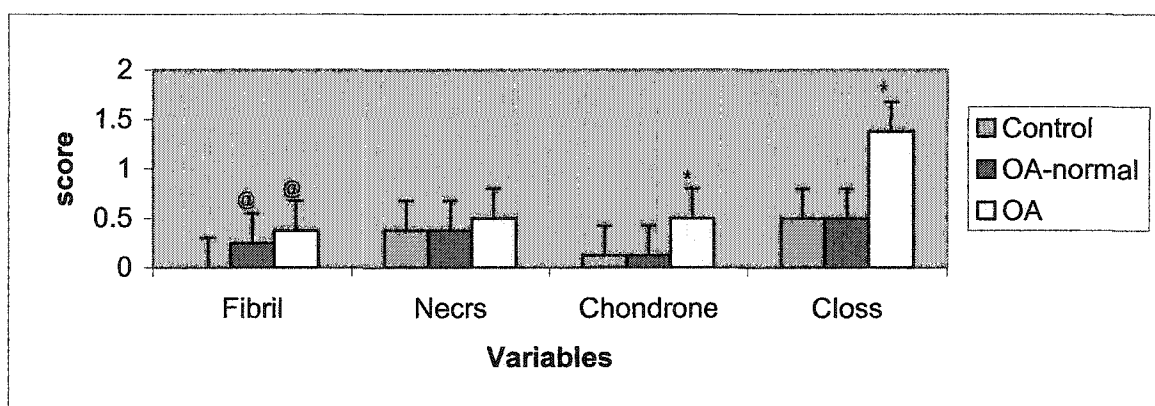


Figure 10. The mean ($\pm$ SEM) scores of histopathologic variables for the articular cartilage sections obtained from the radiocarpal bones and stained with H&E. The scores of chondrone and focal cell loss (Closs) were significantly higher in the OA group compared to the other two groups (*). The fibrillation (Fibril) scores were significantly higher in OA and OA-normal groups compared to the control group (@). No significant differences between groups were noted for cell necrosis (Necrs). $P < 0.05$.

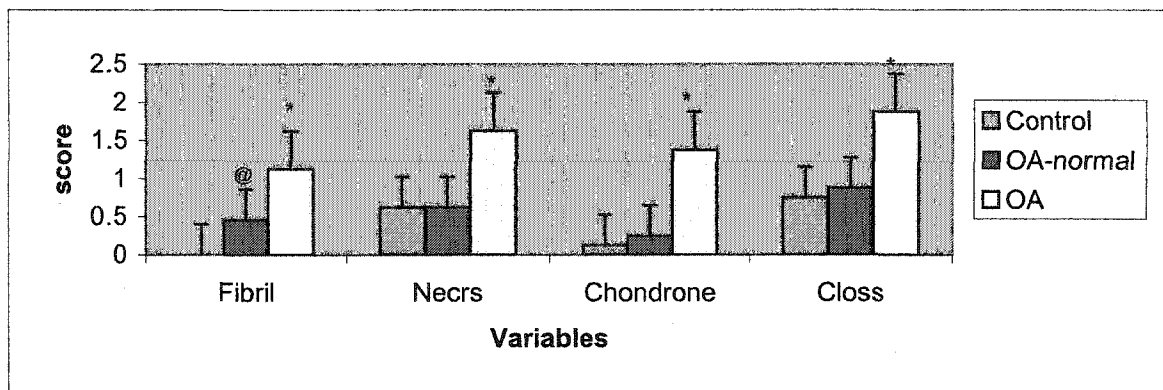


Figure 11. The mean ($\pm$ SEM) scores of histopathologic variables for the articular cartilage sections obtained from the third carpal bones and stained with H&E. The scores of all variables were significantly higher in the OA group compared to the other two groups (*). The fibrillation (Fibril) scores were significantly higher in the OA-normal group compared to the control group (@) ($P < 0.05$).

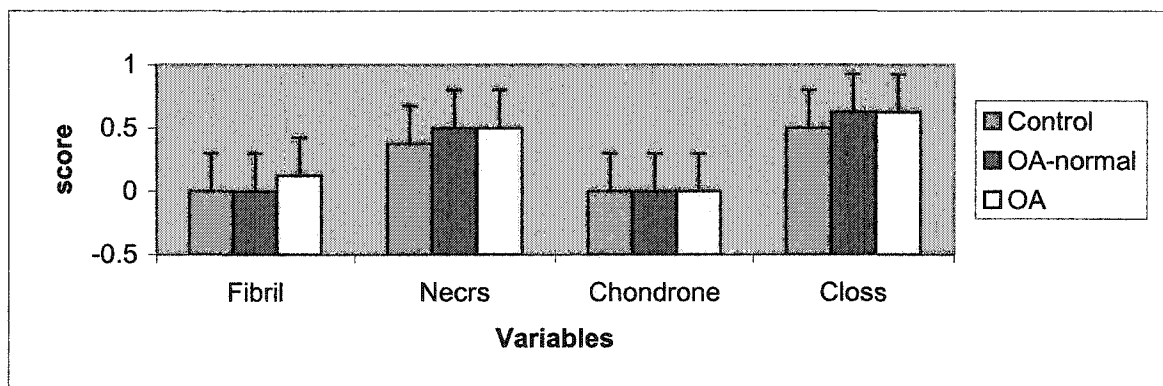


Figure 12. The mean ($\pm$ SEM) scores of histopathologic variables for the articular cartilage sections obtained from the fourth carpal bones and stained with H&E. There were no significant differences among the groups in any of variables at $P < 0.05$.

Table 4. Values of R^2 between articular cartilage histopathologic variables and synovial fluid biomarkers. The * indicates significance at $P < 0.05$.

Biomarker	Fibrillation	Cell necrosis	Chondrone	Cell loss
CS846	0.36*	0.04	0.03	0.49*
CPII	0.46*	0.41*	0.44*	0.01
sGAG	0.53*	0.1	0.04	0.41*
COL23/4C _{short}	0.49*	0.41*	0.58*	0.04
234CEQ	0.48*	0.38*	0.58*	0.04
PGE ₂	0.58*	0.53*	0.52*	0.52*

Table 5. Values of R^2 between articular cartilage histopathologic variables and serum biomarkers. The * indicates significance at $P < 0.05$.

Biomarker	Fibrillation	Cell necrosis	Chondrone	Cell loss
CS846	0.52*	0.07	0.06	0.4*
CPII	0.37*	0.41*	0.45*	0.06
sGAG	0.08	0.04	0.03	0.52*
COL23/4C _{short}	0.07	0.07	0.01	0.01
234CEQ	0.5*	0.05	0.37*	0.04

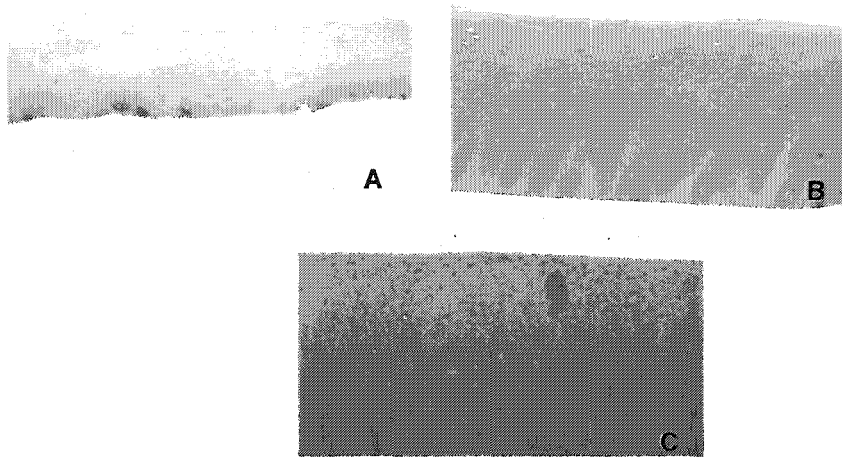


Figure 13 Photomicrographs of 5 μ m sections of articular cartilage stained with SOFG obtained from the third carpal bones for each group. Plate (A) is a representative area of cartilage obtained from the OA group showing no to slight stain uptake pattern. Plate (B) is a representative area of cartilage obtained from the OA-normal group showing moderate stain uptake pattern. Plate (C) is a representative area of cartilage obtained from the control group showing normal stain uptake pattern.

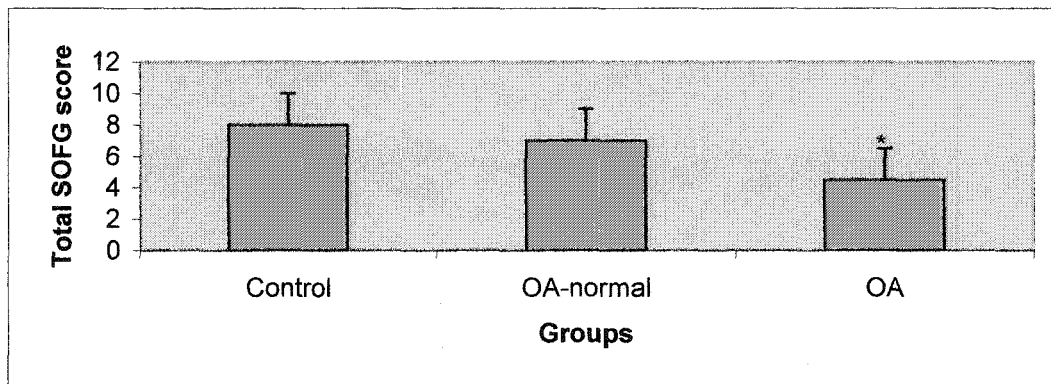


Figure 14. Total score for SOFG in the groups (SEM). The mean total SOFG score in the OA group was significantly lower than those in the other two groups. The significant differences between groups are marked by * ($P < 0.05$).

Table 6. The comparisons between the groups for the safranin O/fast green (SOFG) staining scores of the articular cartilage sections obtained from the radial, third, and fourth carpal bones.

The "Yes" indicates that the scores were significantly lower in the first group compared to the second group ($P < 0.05$). The "No" indicates no significant differences were found between the two groups in the scores at $P < 0.05$. RT = radiate territorial, RIT = radiate interterritorial zones

The bone	The comparison	Tangential	Intermediate	RT	RIT
Radiocarpal bone	OA vs Control	Yes	Yes	No	Yes
	OA-normal vs Control	No	No	No	No
	OA vs OA-normal	Yes	Yes	No	Yes
Third carpal bone	OA vs Control	Yes	Yes	No	Yes
	OA-normal vs Control	Yes	No	No	Yes
	OA vs OA-normal	Yes	Yes	No	Yes
Fourth carpal bone	OA vs Control	No	No	No	No
	OA-normal vs Control	No	No	No	No
	OA vs OA-normal	No	No	No	No

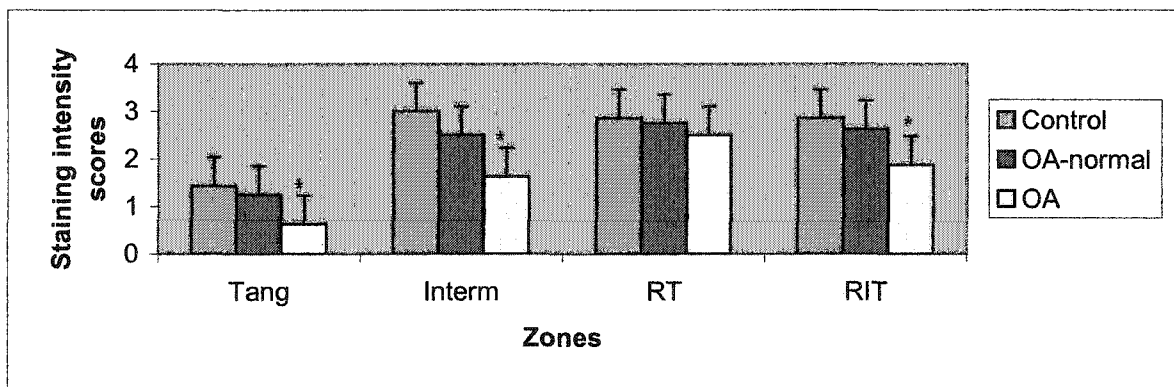


Figure 15. The mean ($\pm$ SEM) staining intensity scores in the different zones of the articular cartilage obtained from the radiocarpal bones and stained by SOFG. The scores of staining intensity in the tangential (Tang), intermediate (Interm) and radiate interterritorial (RIT) zones were significantly lower in the OA group compared to the other two groups (*). ($P < 0.05$). RT indicates the radiate territorial zone.

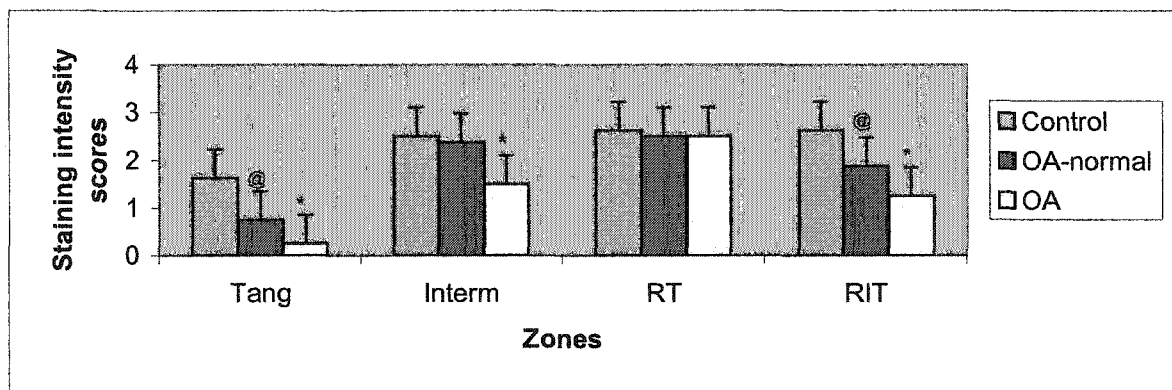


Figure 16. The mean ($\pm$ SEM) staining intensity scores in the different zones of the articular cartilage tissues obtained from the third carpal bones and stained by SOFG. The scores of staining intensity in the tangential (Tang), intermediate (Interm) and radiate interterritorial (RIT) zones were significantly lower in the OA group compared to the other two groups (*). The scores of tangential (Tang) and radiate territorial (RT) zones were significantly lower in the OA-normal group compared to the control group (@). $P < 0.05$. RT indicates radiate territorial zone.

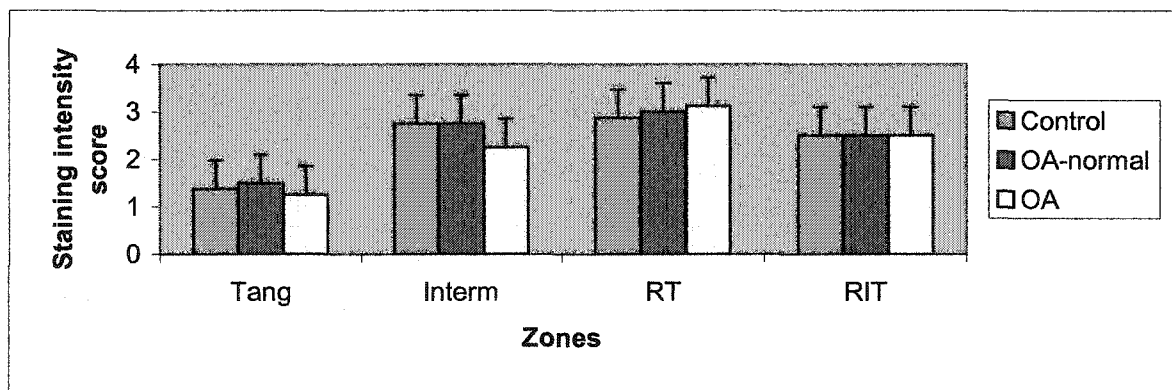


Figure 17. The mean ($\pm$ SEM) staining intensity scores in the different zones of the articular cartilage obtained from the fourth carpal bones and stained by SOFG. There were no significant differences among groups in the staining intensity scores of any zone.

Table 7. Values of R^2 between the SOFG staining scores for the articular cartilage and the synovial fluid biomarkers. Values of R^2 were higher between the SOFG staining scores for articular cartilage zones and the levels of synovial fluid sGAG. The * indicates significance at $P < 0.05$.

Biomarker	Tangential	Intermediate	RT	RI
CS846	0.01	0.01	0.01	0.01
CPII	0.005	0.01	0.008	0.005
sGAG	0.45*	0.47*	0.36*	0.36*
COL2-3/4C _{short}	0.004	0.004	0.006	0.004
234CEQ	0.004	0.0009	0.004	0.005
PGE ₂	0.003	0.008	0.01	0.006

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