Osteoarthritis-

The inflammatory reaction and medical treatment

Anna-Maria Andersson

Supervisor: Stina Ekman
Department of Biomedical Sciences and Veterinary Public Health
Swedish University of Agricultural Sciences

Assistant supervisor: Cecilia Ley
Department of Biomedical Sciences and Veterinary Public Health
Swedish University of Agricultural Sciences

Assistant supervisor: Maija-Leena Eloranta
Department of Medical Sciences
Uppsala University

Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap
Veterinärprogrammet

Examensarbete 2007:24
ISSN 1652-8697
Uppsala 2007

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Sciences
Veterinary Medicine Programme

Degree project 2007:24
ISSN 1652-8697
Uppsala 2007

CONTENTS

SUMMARY

Osteoarthritis is a common and important problem in performance horses and symptoms include lameness accompanied with heat, pain and joint swelling. Several different diagnostic methods exist. However, detection of early stages of inflammation is problematic; hence assays for biochemical markers, such as the cytokines, are considered a possible way to monitor inflammation. Many of the cytokines are mediators released in the process of inflammation and one important example is tumor necrosis factor alpha (TNFα).

In this pilot study of five horses, an ELISA was used to measure TNFα in synovial fluid from fetlock joints. This was performed in order to evaluate if any inflammatory reaction against intra-articularly injected hyaluronan X (hyaluronic acid) was present. Hyaluronan X is a new product with a higher molecular weight compared to hyaluronic suspensions commonly used in equine practice for the treatment of osteoarthritis.

All the horses showed clinical signs of joint inflammation at some point. However, only one of the five horses presented a detectable level of TNFα in synovial fluid from the fetlock joint. Unfortunately, the hyaluronan X had been contaminated with ethanol during the manufacture and hence no conclusions about the side effects of this new hyaluronic suspension could be drawn. In future studies, a revised study design with repeated sampling of synovial fluid from experimental joints as well as control joints must be developed. In addition, the manufacturing of the hyaluronic suspension must be improved.

SAMMANFATTNING

Ledinflammation (osteoartrit) är ett viktigt problem hos arbetande hästar och manifesteras som hälta med associerad värme, smärta och ledsvullnad. Flera olika diagnostiska metoder finns att tillgå men tidiga stadier av inflammation är svåra att upptäcka. Därför analyseras olika biokemiska och inflammatoriska markörer i både ledvätska och serum. Dessa markörer inkluderar de olika mediatorer som frisätts i samband med den inflammatoriska processen i leden och ett viktigt exempel är inflammationscytokinen tumör-nekros-faktor alfa (TNFα).

I den nedan beskrivna pilotstudien med fem hästar injicerades kotleden i ett framben med hyaluronsyra X och den kontralaterala leden användes som kontroll. Hyaluronsyra X är en ny produkt med högre molekylvikt än de som idag används vid behandling av osteoartrit hos häst. En hästspecifik ELISA användes sedan för mätning av TNF_{α} i ledvätska. Tanken med detta var att utvärdera huruvida injektionen av hyaluronsyra X gav upphov till en inflammatorisk reaktion i leden.

Alla fem hästarna i studien visade kliniska tecken på ledinflammation men bara en av dem hade ett förhöjt värde av TNFα i kotleden. Tyvärr var hyaluronsyrasuspensionen vid ett par tillfällen kontaminerad av etanol vilket i sig kan leda till inflammation i leden. Därför kan inga slutsatser om eventuella biverkningar av preparatet dras från denna studie. Inför uppföljande studier bör tillverkningen av hyaluronsyra X förbättras och kvalitetssäkras. Dessutom måste studiedesignen förändras så att upprepade ledvätskeprover tas från både försöksleden och kontrolleden vid samma tillfällen.

ABBREVIATIONS

COX	Cyclooxygenase
ELISA	Enzyme-linked immunosorbent assay
IGF	Insulin-like growth factor
IL	Interleukin
MMP	Matrix metalloproteinases
NO	Nitric oxide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NSAID	Non-steroidal anti-inflammatory drugs
PGE	Prostaglandin E
PMN	Polymorphonuclear leukocytes
PSGAG	Polysulfated glycosaminoglycans
TGF	Transforming growth factor
TIMP	Tissue inhibitors of the matrix metalloproteinases
TNFα	Tumour necrosis factor alpha

INTRODUCTION

Joint disease and associated lameness are common and important problems in performance horses. It represents a welfare issue as well as a source of economic loss due to veterinary expenses, limited performance and early retirements. Therefore, there is a demand for an efficient treatment regime. In search for new diagnostic and treatment possibilities, knowledge about the normal joint and the mechanisms behind osteoarthritis is of huge importance. For successful treatment, there is also a need for a correct diagnosis. In the light of this, there has been a search for improved diagnostic methods. Biochemical markers are an important part of the diagnostic future.

Over the years, various treatments have been used with variable results. However, the perfect treatment is still to be found. The most important medical treatments of today are the non-steroidal anti-inflammatory drugs (NSAID), the glucocorticoids, hyaluronic acid, and the polysulfated glycosaminoglycans (PSGAG). Each one of these has its own specific advantages and disadvantages, and researchers are continuously trying to find a way to improve them. For instance, the short half-life of hyaluronan is an obvious drawback and this was the starting-point for the development of a new hyaluronan suspension.

This suspension, here called “hyaluronan X”, is intended to persist in the joint for a longer time, exert an effect for a longer period of time and thus lead to a better result of treatment. The reason for designing this pilot study was to give the manufacturer an opportunity to investigate *in vivo* how long the suspension actually was present in the joints and, at the same time, control whether there was any side effects associated with the intra-articular injection of the suspension. The aim of my project was to evaluate a possible inflammatory reaction in terms of increased levels of one of the important biochemical mediators, namely tumor necrosis factor alpha (TNFα). This was done through an enzyme-linked immunosorbent assay (ELISA) of synovial fluid.

BACKGROUND

Structure and function of normal diarthrodial joints

A diarthrodial joint consists of two opposing cartilage-covered bones stabilized by peri-articular soft tissues such as muscles, tendons, ligaments and joint capsule (fig 1) (Caron, 2003; Palmer & Bertone, 1994).

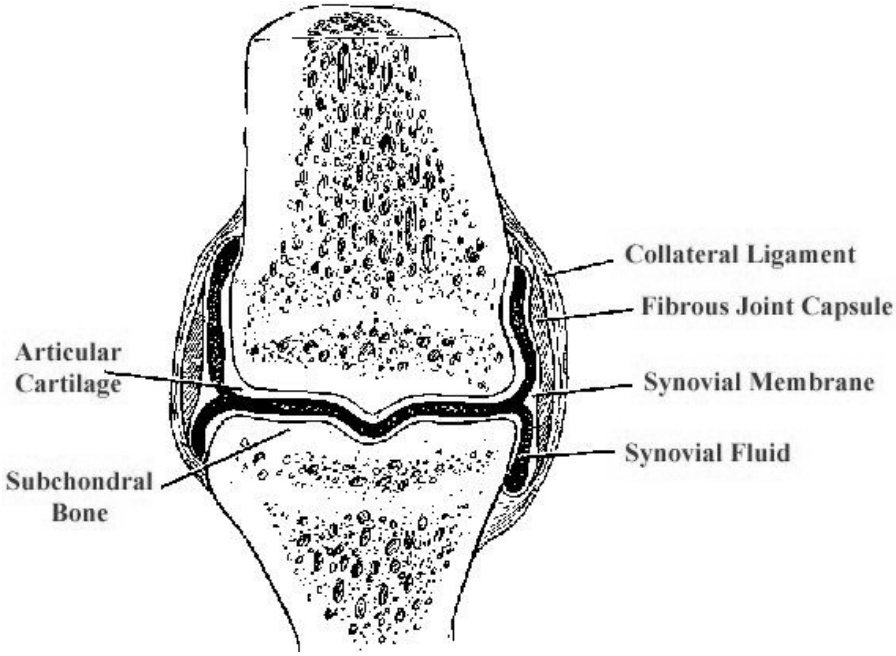

Fig 1: Normal joint structure (McIlwraith, 2006).

Synovium and synovial fluid

The synovium is the vascular connective tissue which covers all the articular surfaces excluding the articular cartilage. It is built up of two layers, the subintima and the intima. The *subintimal layer*, which is adjacent to the fibrous joint capsule, consists of fibrous, areolar and fatty tissues. It is richly supplied by blood vessels and this is essential for the generation of synovial fluid, the exchange of nutrients and metabolic wastes, as well as the nutrition of the avascular articular cartilage. The innermost layer, the *intimal layer*, consists of one to four layers of different synoviocytes (fig 2). First, there are the macrophage-like synoviocytes (type A). Their main task is phagocytosis and they clear the joint of unwanted particles. There are also the fibroblast-like synoviocytes (type B) which are responsible for the production and secretion of macromolecules and proteins involved in the joint metabolism. Examples include hyaluronan, collagen, cytokines, prostaglandin, and proteinases. These are all important in maintaining health but may, if unregulated, be involved in development of disease (Frisbie, 2006b; Caron, 2003).

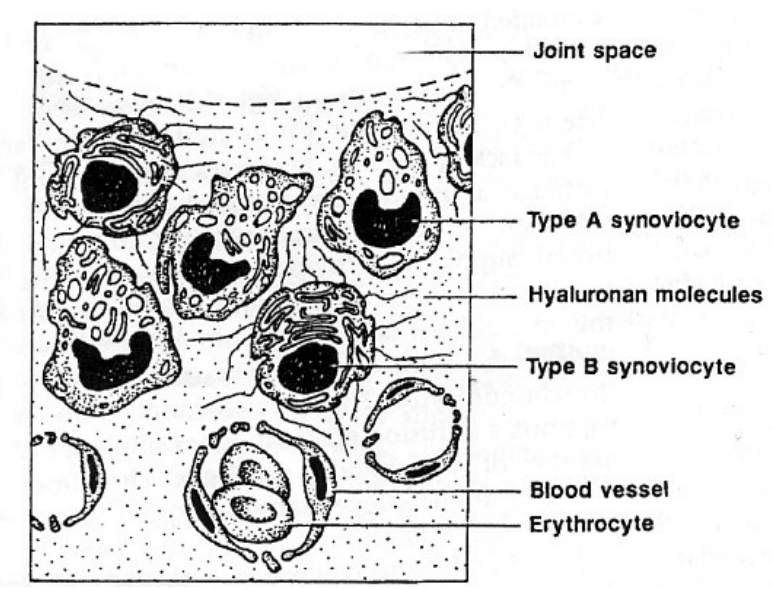

Fig 2: Synoviocytes and hyaluronan molecules (Frisbie, 2006b; page 1038).

The synovial fluid is created through ultrafiltration of plasma. The synovial membrane acts as a selective barrier due to lack of basement membrane as well as lack of junctional complexes between the intimal synoviocytes. Large molecules are excluded while small components of the plasma, less than 10 kDa in size, are allowed through. Examples are glucose, oxygen, carbon dioxide and small proteins. The process is mainly controlled by a complex regulatory system of the synovial blood flow. It is also influenced by other factors such as the molecular composition of the synovial fluid. For instance, size, weight and concentration of hyaluronan are relevant. Other factors involved in the generation of synovial fluid are the degree of inflammation as well as the lymphatic drainage (Frisbie, 2006b; Caron, 2003; Howard & McIlwraith, 1996; Palmer & Bertone, 1994).

Articular cartilage

The articular cartilage is an important structure for the function of the joint. In conjunction with the synovial fluid, it contributes to weight-bearing and almost friction-less motion (Caron, 2003). It covers the subchondral plate of the articular bones and is normally 1-4 mm thick. However, the thickness varies by joints, different locations within them as well as age of the horse (Frisbie, 2006b). The hyaline type of articular cartilage is what predominates in mammals. It is characterized in adults by the lack of innervation, vascularization and lymphatic drainage. It is therefore dependent on nourishment through diffusion from the synovial fluid (Frisbie, 2006b; Ray et al, 1996).

The cartilage is built up of a small percentage of chondrocytes surrounded by extracellular matrix. The latter consists mainly of water (65-80%; freely exchangeable with that of the synovial fluid), collagens (10-30%), and proteoglycans (5-10%) (Caron, 2003). Microscopically, the cartilage can be divided into four layers (fig. 3). The *superficial (tangential) zone* consists of elongated chondrocytes within collagen fibrils aligned parallel to the articular surface. In the *mid (intermediate) zone,* the chondrocytes are larger and more round. They are embedded, single or paired, in collagen fibrils in a random

pattern. The *deep (radial) zone* contains even larger chondrocytes, vertically arranged and contained in radially arranged collagen fibrils. The most basal layer is the *zone of calcified cartilage* consisting of chondrocytes dispersed in a mineralised matrix and separated from the non-calcified cartilage by the so-called tide line (Frisbie, 2006b; Ray et al, 1996).

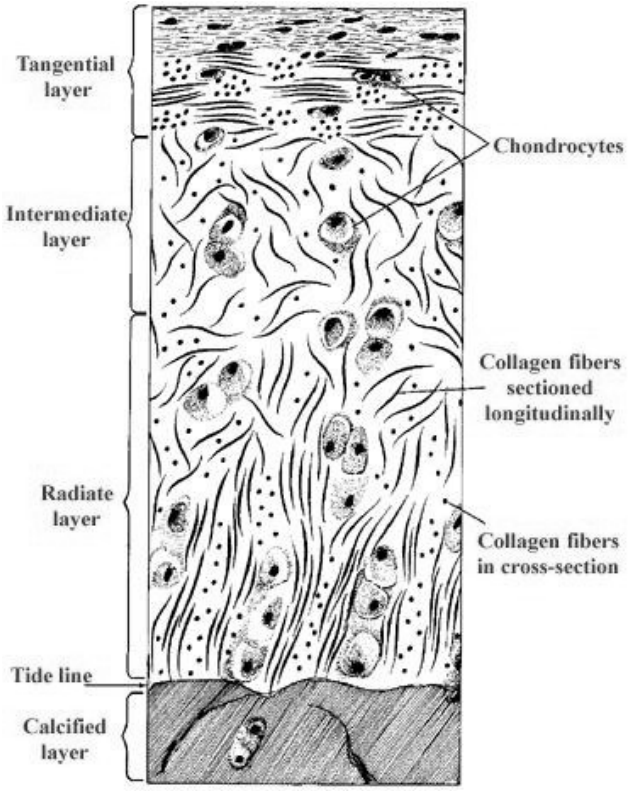

Fig 3: The different layers of articular cartilage (McIlwraith, 2006).

The chondrocytes are responsible for the synthesis of extracellular matrix and serve to balance the proteolytic enzymes involved in the degradation of matrix macromolecules. In a healthy joint, there is a homeostatic relationship in a complex interaction between chondrocytes, biochemical mediators and mechanical stimuli. The cartilage loss in osteoarthritis can be ascribed to a disequilibrium leading to matrix degradation (Caron, 2003; McIlwraith, 1996).

Many different collagens, both fibrillar and non-fibrillar, are found in equine cartilage and they provide a structural framework and give tensile strength (Frisbie, 2006b; Caron, 2003). Briefly, the function of the collagens depends on the zone; in the superficial zone, the main function is protection whereas in the deeper layers the collagen instead anchors the cartilage to the underlying bone (Caron, 2003). The most abundant collagen, accounting for 90%, is type II which is synthesized by and secreted from the chondrocytes. It is built up of cross-linked fibrils consisting of triple helices of identical amino acid chains (Ray et al, 1996). The rate of normal turnover of collagen is significant during growth but extremely slow in adults. The collagen turnover is mediated by collagenase secreted by chondrocytes. The collagenase is proteolytically cleaved in order to become active and further control of the collagenase activity occurs by specific inhibitors (Caron, 2003; Palmer & Bertone, 1994).

The proteoglycans consist of a protein core with glycosaminoglycans (glycoproteins) attached to it. The largest and most abundant of the proteoglycans in articular cartilage is aggrecan. It is characterized by its ability to bind to hyaluronic acid by a non-covalent binding stabilized by a link protein (Frisbie, 2006b; Ray et al, 1996). The glycosaminoglycans associated with aggrecan, chondroitin sulphates and keratan sulphate, contain carboxyl and sulphate groups which make the aggrecan highly negatively charged and thus able to bind huge amount of water. This makes the cartilage resistant to compressive forces (Caron, 2003). The rate of turnover is faster than that of the collagens and, in normal cartilage, the synthesis by the chondrocytes is balanced to the degradation by extracellular proteinases (McIlwraith, 1996).

Traumatic arthritis

Arthritis in athletic horses typically develops after single or repetitive episodes of trauma. Generally, joint tissues are more resistant towards shear forces than repetitive trauma; a central etiologic factor is therefore the mechanical stress associated with exercise. The joints mostly affected are the carpal, fetlock, coffin and tarsal joints. Lameness accompanied with heat, pain and synovial effusion are common clinical signs (Caron, 2003; Howard & McIlwraith, 1996). The viscosity of synovial fluid decreases both due to depolymerisation of the hyaluronic acid as well as dilution of the synovial fluid because of inflammatory effusion (Lindholm et al, 1996).

Traumatic arthritis, according to Howard & McIlwraith (1996), represents a collection of pathologic and clinical states. It includes components such as synovitis, capsulitis, direct articular damage, or subchondral bone lesion which, each and all, may progress into osteoarthritis. In addition, there is an interrelationship between them. The synovial fluid of joints with either one of these conditions contains endogenous products that may be responsible for the initiation or continued progression of any of the others. Examples of such endogenous products are prostaglandins, free radicals, cytokines and proteoglycan fragments. The pain, joint effusion, and decreased range of motion are all consequences of the inflamed synovium and joint capsule (Howard & McIlwraith, 1996; Lindholm et al, 1996).

Osteoarthritis

The term osteoarthritis describes a disorder of joints in which degeneration and loss of articular cartilage is a central feature (fig 4). Other terms exist in order to describe the same phenomena such as degenerative joint disease (Frisbie, 2006b).

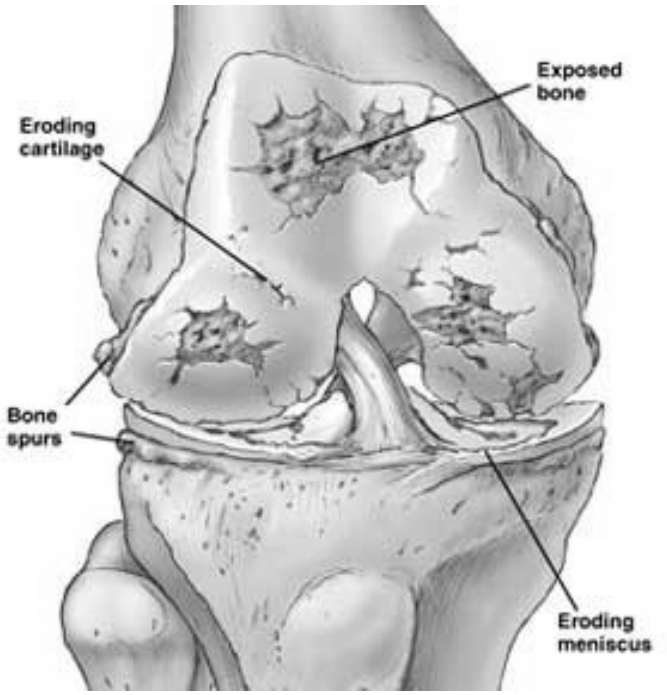

Fig 4: Osteoarthritis (Pain Relief Cushions, 2006).

Ethiopathogenesis

Osteoarthritis is not a single disease but rather a group of biomechanical and biochemical mechanisms leading to synovitis and cartilage detoriation (Trumble et al, 2001). Different authors have tried to sort this out by categorising the factors involved.

Equine osteoarthritis may be classified due to the *factors initiating* the disease. One category of cases is usually preceded by synovitis and capsulitis and is typically seen in joints such as the carpus, fetlock, and distal tarsal/ interphalangeal joints. Another type is associated with identified injuries including intra-articular fractures, osteochondrosis, subchondral bone injury, and septic arthritis. There are also cases that appear to be associated with incidental or non progressive articular cartilage erosion (McIlwraith, 1996).

Three different *pathogenetic mechanisms* of osteoarthritis have been described (Caron, 2003; McIlwraith, 1996):

- A fundamentally defective cartilage with abnormal biomechanical properties which fails under normal loading conditions. Possible etiological factors to the defective cartilage are age, osteochondrosis as well as trauma leading to joint inflammation and subsequent degeneration of the articular cartilage.
- A normal cartilage which is exposed to abnormal mechanical forces. Under normal conditions, the articular cartilage remains healthy despite being subjected to large forces. This is mainly due to transmission of forces to the peri-articular tissues, the congruity of cartilage surfaces and the compliance of cartilage and subchondral bone. However, in cases of joint incongruity (developmental defects), loss of stability (ligamentous tears) or supraphysiological repetitive load, the cells and the extracellular matrix of the cartilage may be damaged.
- An alternative mechanism of cartilage destruction is present when the subchondral bone is impaired with sclerosis or lytic changes. Normally, mechanical load leads to microfractures which are continuously healing. However, when the microfractures occur at an increased frequency, the rate of optimal healing and remodeling is exceeded and the subchondral bone plate and trabeculae increase in density. This results in a pathological sclerosis with a less compliant bone, prone to failure, and subsequent supra-physiological stress of the articular cartilage even at a normal load. As a consequence, the cartilage may be mechanically damaged.

A more simple classification is the one offered by Trumble and co-workers (2001). They classify osteoarthritis as either *primary or secondary* with primary being the result of cumulative stress (repetitive trauma) and secondary a result of preexisting tissue abnormality (osteochondral fragmentation).

Synovial membrane and its role in disease

The synovial membrane may be damaged by high biomechanical load such as repeated trauma, instability of the fibrous capsule, or abnormalities in the joint. However, it may also be damaged in the absence of high biomechanical load. An example is increased intra-articular pressure resulting in impaired circulation in synovial capillaries. In addition to a primary decrease in oxygen tension in the joint, a possible consequence to the ischemia is a so-called reperfusion injury. This occurs due to the formation of xanthine oxidase from xanthine reductase in the conversion of ATP to hypoxanthine during ischemia. When the joint relaxes and reperfusion occurs, the hypoxanthine reacts with oxygen under influence of xanthine oxidase and the result is uric acid and oxygen-derived radicals. These radicals are then capable of damaging both cellular and matrix macromolecules (Frisbie, 2006b; McIlwraith, 1996).

The damaged synovial membrane is an important source of inflammatory mediators (Frisbie, 2006b; Caron, 2003). It is also a source of pain due to stimulation of pain receptors in the capsular tissues and in the subchondral bone (McIlwraith, 1996).

Articular cartilage degradation

The articular cartilage degeneration depends on the fact that, in osteoarthritis, the chondrocytes are unable to maintain the homeostasis in the matrix and the consequence is net matrix degradation. Macroscopically, this can be seen as fibrillation, erosions and wear lines in the articular cartilage. Upon compression, the cartilage is softer than normal. Superficial fibrillation, chondrone formation, chondrocyte necrosis and full-thickness loss of cartilage are histological characteristics. Other common findings are subchondral bone sclerosis, subchondral cystic lesions, penetration of the tidemark by blood vessels, focal osteonecrosis, and periarticular osteophyte formation (McIlwraith, 1996).

The loss of proteoglycans is an important initial biochemical change in osteoarthritis (Caron, 2003). The up-regulation of proteoglycan synthesis by the chondrocytes is insufficient and thus, as the inflammation proceeds, there is a decreased concentration of proteoglycans in all layers of the articular cartilage (Ray et al, 1996). The proteoglycan loss is accompanied by a degradation of collagen (Caron, 2003). Other important biochemical findings are changes in the degree of proteoglycan aggregation as well as increased water content of the cartilage (Moreland, 2003; McIlwraith, 1996). This results in weakening of the cartilage matrix, loss of viscoelastic properties and subsequent failure under normal loads (Caron, 2003).

Upon damage, there is a limited potential of articular cartilage to react in order to produce tissue with the same morphological, biochemical and biomechanical properties. Three mechanisms by which cartilage may repair are (i) *intrinsic repair* with the limited mitotic capability of the chondrocyte and its minor increase in collagen and proteoglycan production, (ii) *extrinsic repair* in which cartilage is formed through metaplastic change of mesenchymal components of the subchondral bone, and (iii) by *matrix flow* in which cartilage is formed at the edge of the articular cartilage defect and then migrate toward the centre. Several factors contribute to the repair process such as depth of the injury, size of the defect, location and relation to weight-bearing/non-weight-bearing areas as well as age of the animal (McIlwraith, 1996).

In addition to the articular cartilage breakdown, there may be proliferation of new cartilage and bone at the joint periphery (so called osteophytes; fig 4 “bone spurs”). Enthesophytes are bone proliferations at the insertions of ligaments, tendons, or joint capsule. There are several predisposing factors which cause osteophyte development such as age, mechanical instability, and synovitis. It is important to know that osteophytes can be seen in horses without articular cartilage damage and without clinical significance (McIlwraith, 1996).

Mediators of inflammation and cartilage destruction

The mediators of joint inflammation can be divided into two groups according to their availability and function; the pre-formed mediators and the induced mediators (Palmer & Bertone, 1994).

The *pre-formed mediators* infiltrate the joint following traumatic injury to the synovial membrane and disruption of the blood-synovial membrane. This is preceded by the entrance of latent forms of non-specific mediators of inflammation such as kinin, histamine, products of the complement pathway as well as components of the blood coagulation and fibrinolytic systems. These act as chemoattractants for leukocytes. All potential proteins released from the granules of these infiltrating leukocytes are included in this group of pre-formed mediators. The primary granulae are a source of lysozyme, cathepsins, and serine proteinases and from the secondary granulae spring lysozyme, proteinases, collagenase and gelatinase (Palmer & Bertone, 1994).

All of these mediators are capable of contributing to changes in the synovial fluid, and to some extent also to cartilage destruction. The influence on cartilage destruction is, however, limited due to their large size and subsequent limited potential of diffusion into cartilage. The lysozyme released is involved in the degradation of hyaluronic acid while the serine proteinases affect the proteoglycan content in the cartilage: Elastase, for instance, may cleave the protein core at a number of sites. Cathepsin G instead cleaves the largest of the link proteins and the hyaluronic acid is therefore disaggregated from the proteoglycan. In addition, the changes in the synovial fluid lead to changes in lubrication, pH and nutrition (Palmer & Bertone, 1994).

Leukocyte-derived substances also stimulate macrophages, synoviocytes and chondrocytes; the result is synthesis of the so-called *induced mediators* such as cytokines, eicosanoids and matrix enzymes. These are significantly more important in the destruction of cartilage matrix since they can have an effect on the cellular metabolism as well as the activation or inhibition of enzymes. What determines the outcome of the inflammatory process in the end, however, is the balance of these induced mediators with their inhibitors as well as the cellular response. If the inflammation is halted and reversed, continued damage to the joint surface is unlikely. However, if the inflammation persists, it will alter the nutritional supply to the cartilage and the presence of mediators will continue the inflammation and cartilage degradation (Palmer & Bertone, 1994).

Cytokines are a group of soluble peptides whose production is induced via transcriptional activation in stimulated cells. They are produced by one cell and exert an effect on another through an endocrine, paracrine or autocrine manner. By binding to specific trans-membrane receptors, they affect gene expression of the target cell either positively or negatively. A third characteristic of cytokines is that they are able to influence the production and action of other cytokines via specific receptors (McIlwraith, 1996; Palmer & Bertone, 1994). In joints, they are important for the regulation of the metabolism of the synovial membrane, bone, and articular cartilage (Caron, 2003).

The cytokines has been discussed as being catabolic, modulatory or anabolic (Frisbie, 2006). The two most important *catabolic* (pro-inflammatory) cytokines are interleukin 1 (IL-1) and tumour necrosis factor alpha (TNFα). These are secreted from chondrocytes and synoviocytes, and have been shown to be up-regulated in osteoarthritic joints (Caron, 2003; McIlwraith, 1996). IL-1 and TNFα have similar effect in the process of inflammation and destruction of joints, and act synergistically (Frisbie, 2006; Billinghurst et al, 1995). They contribute to joint destruction by promoting the production of other induced mediators such as eicosanoids and matrix metalloproteinases (MMPs). In addition, they inhibit the synthesis of aggrecan and collagen (Frisbie, 2006; Hawkins et al., 1993) as well as the production of the tissue inhibitors of the matrix metalloproteinases (TIMP) (Caron, 2003). Further, IL-1 affects the collagen synthesis resulting in formation of functionally inadequate repair tissue (McIlwraith, 1996; Ray et al, 1996). The *modulatory* cytokines include interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 13 (IL-13). They inhibit the synthesis of IL-1 and promote the synthesis of TIMP. Interleukin 6 (IL-6), another cytokine belonging to this group, has a mixed mode of action and both potentiate the effects of IL-1 and promote the synthesis of TIMP. The production of IL-6 is mainly stimulated by the catabolic cytokines IL-1 and TNFα (Frisbie, 2006; McIlwraith, 1996; Palmer & Bertone; 1994). Finally, the *anabolic* (anti-inflammatory) cytokines include insulin-like growth factor (IGF) and transforming growth factor (TGF). IGF-1 is capable of decreasing the degradation and promoting the synthesis of matrix molecules. TGF-β may stimulate the production of TIMP, inhibit the production of MMPs (Ray et al, 1996) and inhibit IL-1-induced effects by decreasing the expression of the IL-1 receptor (McIlwraith, 1996; Palmer & Bertone, 1994).

IL-1 has a unique effect compared to the other cytokines, namely the capacity to activate phospholipase A_2 and therefore stimulate the arachidonic acid cascade (fig 5) (Palmer & Bertone, 1994). One important arachidonic acid metabolite in osteoarthritis is the eicosanoid prostaglandin E_2 (PGE_2) which is produced by the chondrocytes as a response to IL-1 and to mechanical trauma. PGE_2 causes a decrease in the proteoglycan content of cartilage matrix. Vasodilatation, enhancement of pain perception, and bone demineralisation are other actions of PGE_2 in joints. The concentration of PGE_2 is low in normal joints but has been shown to increase in joints with osteoarthritis and lameness (McIlwraith, 1996; Palmer & Bertone, 1994).

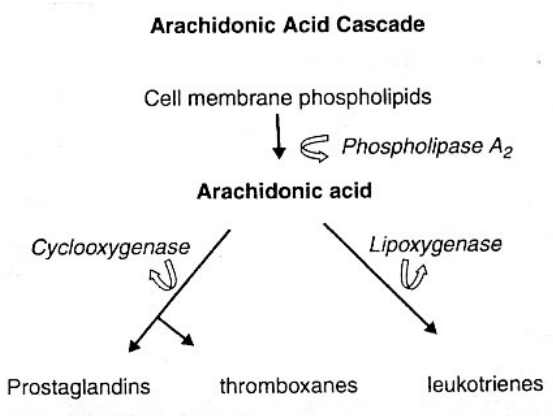

Fig 5: Arachidonic acid cascade (Caron & Genovese, 2003, page 746).

The major mediators of articular cartilage matrix depletion are considered to be the MMPs (fig 6), stemming from the matrix as well as from synovial membrane, blood, synovial fluid, and bone. The MMPs are characterised by a requirement for Zn^{2+} and Ca^{2+} (Caron, 2003; McIlwraith, 1996). They are synthesized by synoviocytes and chondrocytes, and are found in increased concentrations in diseased cartilage. Due to their ability to digest all major components of the extracellular matrix, they play a major role in articular cartilage matrix degradation (Frisbie, 2006). There are many different MMPs in the articular tissues and they may be divided into classes according to the type of cartilage substrate that they degrade. For instance, the *collagenases* cleave intact type II collagen, the *stromelysins* digest partially degraded collagen, proteoglycans, and other minor cartilage proteins, and the *gelatinases* degrade a broad spectrum of substrates (Caron, 2003). In healthy cartilage, the activity of MMPs is controlled at a number of levels. Besides control of synthesis and secretion, the MMPs are secreted as latent proenzymes and require extracellular proteolytic cleavage by a variety of proteinases in order to become active. Further, the MMP activity is regulated through direct inhibition by TIMP. In the normal articular tissues, most MMPs are present in an inactive latent or inhibited form. However, as some studies indicate that there is a lack of TIMP in osteoarthritis and an elevated activity of MMPs such as collagenase and stromelysin (Caron, 2003; Trumble et al., 2001; McIlwraith, 1996).

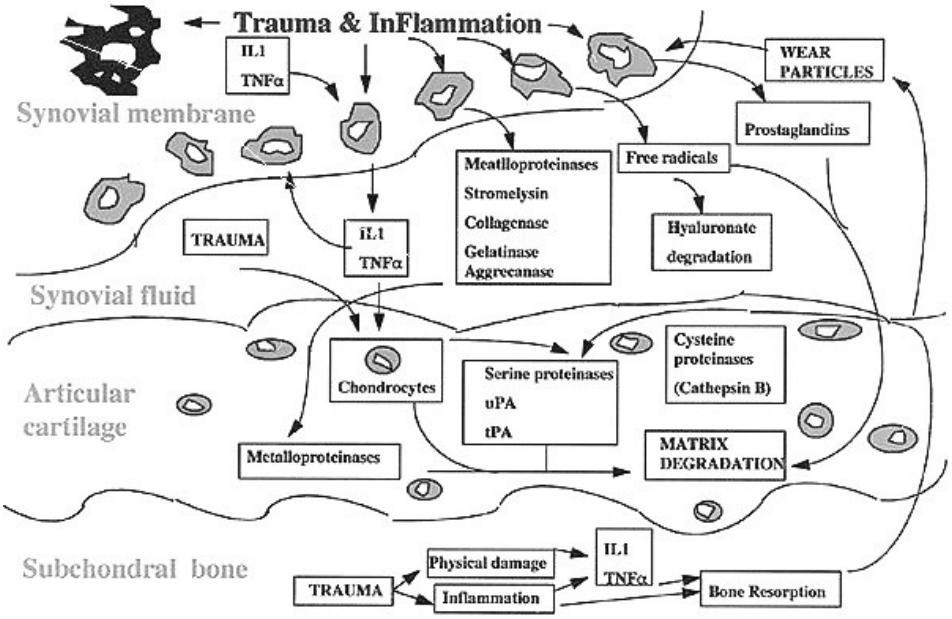

Fig 6: Mediators of articular cartilage matrix degradation (McIlwraith, 2006).

In addition to these mediators, oxygen-derived free radicals are produced during the process of inflammation by NADPH-oxidase in the cell membranes of polymorphonuclear leukocytes (PMNs) and macrophages. One important example is hydrogen peroxide. These radicals are capable of causing damage to the molecules and cells in the proximity. In the joint, a direct damage to the articular cartilage matrix with degradation and inhibited synthesis of proteoglycan can be noted. In addition, an indirect damage by inactivation of TIMP and depolymerisation of hyaluronic acid is reported (McIlwraith, 1996; Palmer & Bertone, 1994).

Finally, worth mentioning are nitric oxide (NO) and the neuropeptides. NO is created in the oxidation of L-arginine upon stimulation by endotoxins and cytokines. Its proinflammatory actions include vasodilatation, increased vascular permeability as well as stimulation of the production of prostaglandins (Rang et al., 2003a). NO also inhibits the synthesis of proteoglycan and collagen, and enhances the MMP activity. In addition, NO is capable of inducing apoptosis of chondrocytes which contributes to reduced matrix production (Caron, 2003; Moreland, 2003). The neuropeptides, such as substance P, are important in pain sensation and also involved in the pathogenesis of the disease. For instance, when monocytes are exposed to substance P the result is release of the cytokines IL-1, IL-6, and TNFα (Rang et al., 2003a; McIlwraith, 1996).

Markers of joint disease

The diagnosis of equine lameness and osteoarthritis has historically relied on clinical symptoms and radiological examination. However, radiological changes are visible only in advanced stages of disease and poorly correlated with clinical signs, synovitis and cartilage degeneration. Therefore, researchers are trying to find biological markers, such as inflammatory mediators or products from articular cartilage destruction, in synovial fluid and serum which would be helpful in diagnostics. These markers would make it possible to detect early changes, preferably before any structural damage has occurred, as well as mild chronic joint disease or cartilage destruction prior to clinical lameness. This would open new possibilities for evaluation of different treatment regimes and hence render a more accurate prognosis (Van den Boom et al, 2004; Bertone et al., 2001; Ray et al., 1996; Billinghurst et al, 1995).

TNFα is one possible marker of joint inflammation. A higher concentration of TNFα in synovial fluids from joints with acute severe joint disease compared to normal joints has been found (Bertone et al., 2001; Billinghurst et al, 1995). However, this increase is not present in degenerative joints in general (Billinghurst et al, 1995), and a correlation between TNFα activity in synovial fluids and degree of joint damage has not been found (Jouglin et al., 2000). The conclusion from the presented research, so far, is that an increase in the concentration of TNFα in synovial fluid is a good predictor of acute arthritis. In these cases, the presence of synovitis is partly responsible for the increase of TNFα. Chronic osteoarthritis or cases with a cartilage defect but no synovitis fail to correlate with TNFα values (Bertone et al., 2001). The peak of TNFα level has been found to occur early in the process of experimentally induced inflammation and some researchers report as early as 2-4 h after onset of inflammation (Cornelissen et al., 1998; Billinghurst et al, 1995; Hawkins et al., 1993).

Other possible markers, such as IL-6 and PGE_2, have been proposed for detecting the presence of joint disease but the research, so far, has not managed to correlate the cytokine levels with specific joint pathology. IL-1, as TNFα, is a good predictor in acute severe cases but cannot adequately detect joint disease in general. On the other hand, $PGF_{1\alpha}$ has high sensitivity and specificity for chronic cases. However, since severe and/or chronic cases of osteoarthritis often may be diagnosed with already existing means (clinical examination, radiology, and ultrasound), these biochemical markers are of limited use (Bertone et al., 2001).

Medical treatment of joint disease

Treatment of equine osteoarthritis serves two aims; symptomatic relief and arresting or slowing the progression of joint degeneration. There are different ways of administrating the drugs; oral intake or injections (intra-muscular, intra-venous or intra-articular).

NSAID

The role of the non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of osteoarthritis is attributable to the fact that they are relatively inexpensive and generally effective in reducing lameness (Caron, 2005). Further more, they are easily administrated in the form of oral preparations. Intervention of the arachidonic acid cascade (inhibition of cyclooxygenases COX-1 and/or COX-2; fig 5) and subsequent inhibition of the production of prostaglandins and thromboxanes are the main actions of NSAIDs (Rang et al., 2003a). Traditionally, this inhibition and the following pain relief have been considered entirely positive (Frisbie, 2006a). However, the actions of the prostaglandins are not entirely clear and there is evidence that they ambiguously also play a role in the inhibition of the MMPs. Thus, treatment with NSAIDs may actually increase the activity of the MMPs. In addition, promotion of injury may also occur through inhibition of chondrocyte metabolism or indirectly through increased weight-bearing due to pain relief. This means that the effects of NSAIDs may, in the long run, instead be deleterious. Importantly, there is a considerable variation between the different NSAIDs concerning the exact effects and side-effects (Caron and Genovese, 2003; Palmer & Bertone, 1994).

Glucocorticoids

The glucocorticoids are potent anti-inflammatory agents widely used to treat osteoarthritis. They are capable of depressing many different inflammatory processes. Generally, the effects of the glucocorticoids are mediated through interactions with intra-cellular receptors resulting in changes in gene transcription. Repression of genes, for instance, results in inhibition of transcription factors normally involved in the production of COX-2 (and subsequently prostaglandin), cytokines (such as the interleukins), MMPs (e.g. collagenase), and NO. Induction of genes, instead, leads to formation of annexin-1. This protein has anti-inflammatory actions through inhibition of phospholipase A_2 (affecting the production of prostaglandin) (Rang et al., 2003b). In summary the glucocorticoids, in comparison to the NSAIDs, inhibit the production of prostaglandins by inhibiting phospholipase A_2 and selectively COX-2 (fig 5) (Frisbie, 2006a; Rang et al., 2003a). Other anti-inflammatory effects include reduced influx and decreased activity of leukocytes. In addition, due to decreased vasodilatation and fluid exudation, the glucocorticoids are capable of preventing joint swelling (Rang et al., 2003b; Creamer 1997).

Due to the risk for systemic toxic effects after oral administration, the glucocorticoids are normally injected directly into the affected joints and the suspensions generally have short intra-articular half-lives. One factor assumed to enable better penetration of intra-articular tissues and thus increase the duration of the clinical response is a period of rest following injection (Caron, 2005). As with

the NSAIDs, differences in the duration and therapeutic effects of the different preparations exist which make them suitable for different type of joints and joint lesions (Frisbie, 2006; Caron & Genovese, 2003).

The importance of glucocorticoids in treatment of osteoarthritis is controversial. It has been shown that, at low doses, the glucocorticoids have chondroprotective properties due to the diminished concentration of inflammatory mediators otherwise leading to cartilage degradation. However, at high concentrations, the glucocorticoids may instead be deleterious due to suppression of proteoglycan synthesis and influence on the structural organization of collagens (Caron & Genovese, 2003). These changes are largely reversible and some researchers claim that inflamed joints do not exhibit the detrimental effects observed in normal joints after corticosteroid administration (Frisbie, 2006a; Caron 2005; Todhunter, 1998) Thus, Caron & Genovese (2003) argue that the risks of corticosteroid treatment probably are overemphasized.

Hyaluronan

Hyaluronic acid is a linear non-sulfated glycosaminoglycan mainly synthesized by the synoviocytes and the chondrocytes (fig 7). It is anionic and consequently associated with various cations under physiologic conditions. Therefore, more correctly, it is referred to as hyaluronate or hyaluronan in cases with an undetermined cation (Howard & McIlwraith, 1996).

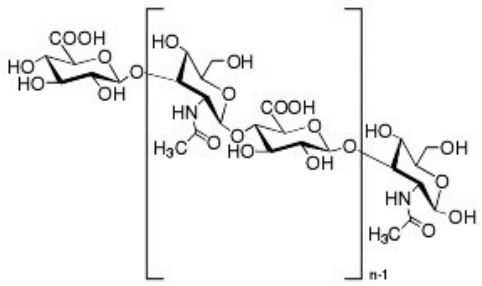

Fig 7: Hyaluronic acid (Läkemedelsindustriföreningen, 2006).

Many researchers have described the physical properties of hyaluronan. Depending on the specific method used, the molecular mass of synovial fluid hyaluronan has been estimated to be in the range of 1 to 6 * 10^6 Da. The concentrations registered are between 0.33 to 1.5 mg/ml. Variations exist between species, between joints of the individual animal (with the smaller joints generally exhibiting a higher concentration) as well as between methods used (Howard & McIlwraith, 1996).

Hyaluronan is a component of synovial fluid as well as of the proteoglycan aggregates of articular cartilage in normal joints (Howard & McIlwraith, 1996). It is involved in the maintenance of synovial homeostasis and viscoelasticity (Caron & Genovese, 2003). Different studies have indicated that reduction in size and synovial fluid concentration of hyaluronan accompanies the disease. Fragmentation of hyaluronic acid is mediated through IL-1/TNFα stimulated hyaluronic acid synthetase (Moreland, 2003; Howard & McIlwraith, 1996).

Preparations exist for administration either intramuscularly, intravenously or directly into the joint; with the latter being the most studied. One mechanism of action of exogenously administrated hyaluronan concerns its *anti-inflammatory effect*; receptor-mediated or via physical mechanisms (Caron, 2005). This anti-inflammatory effect can be attributed to reduced production of prostaglandins as well as to decreased production, reduced activation and increased inhibition of different other inflammatory mediators. Further more, the possibility of physical hindrance results in prevention of diffusion of deleterious substances into the cartilage matrix and subsequent decrease in interaction of enzymes, antigens and cytokines with their target cells. In addition, potential anti-inflammatory effects such as inhibition of chemotaxis, migration and phagocytosis of inflammatory cells have been demonstrated (Moreland, 2003; Howard & McIlwraith, 1996).

A complementary mechanism of action has been described. This is the *matrix protective effect* due to stimulation of proteoglycan synthesis by the chondrocytes and a blocked release of proteoglycans into the synovial fluid (Caron & Genovese, 2003; Moreland, 2003; Palmer & Bertone, 1994). More importantly, hyaluronan may also protect the matrix from the catabolic effects of IL-1 and other mediators (Caron, 2005; Moreland, 2003). Finally, any improvement of health and function of the articular soft tissues in general should lead to a more physiologic joint environment and indirectly reduced cartilage degeneration (Howard & McIlwraith, 1996).

Hyaluronan has an effect on the *nociception* due to decrease of hyperanalgesic nerve activity and attenuation of PGE_2. In addition, hyaluronan has an effect on substance P. Further, treatment with hyaluronan may also *improve synovial fluid viscosity* and intra-articular soft tissue lubrication with an associated increase in range of motion (Caron, 2005; Caron & Genovese, 2003). Cross-linked exogenous hyaluronan has been developed for this purpose (viscosupplementation). Compared to other synthetic hyaluronan suspensions, it seems to have a longer retention time in the synovial cavity (Frisbie, 2006a).

Due to short turn over rates, it is concluded that the effects on synovial and cartilage metabolism must be indirect (Caron & Genovese, 2003). Estimations of the half-life of exogenous hyaluronan injected into normal equine joints have varied; Popot and co-workers (2004) suggest it to be close to 5 hours while Lindholm and co-workers (1996) reported a half-life of between 9 – 16 hours depending on the joint studied. In diseased joints, the half-life is suggested to be shorter. Most of the administrated hyaluronan is cleared from the joint but a part of it remains associated with different synovial tissues and may, for instance, influence the metabolic activities of the synoviocytes (Howard & McIlwraith, 1996). In addition, it has been shown that injection of hyaluronan also may stimulate synthesis of endogenous hyaluronan by the synoviocytes (Caron & Genovese, 2003; Moreland, 2003) leading to a more long-lasting effect.

The effect of the molecular weight on efficacy of hyaluronan is still controversial. The correlation between molecular weight and clinical effect is not clear even though several *in vitro* experiments have indicated a molecular weight-dependent effect (Howard & McIlwraith, 1996). Aviad and co-workers (1994) suggest that the therapeutic effect of hyaluronan is mediated through cellular modulation and therefore it is not the physical properties such as molecular weight that are

important. However, other studies have indicated that a basal molecular mass is needed for effectiveness. This basal mass has been suggested to be $1 * 10^6$ Da (Caron & Genovese, 2003). Another study showed a significant difference in the duration of soundness in horses treated with preparations of hyaluronan with a molecular mass > $2 *10^6$ Da (Philips, 1989). Unfortunately, some other studies have demonstrated better results with hyaluronan of lower molecular mass and hence Caron (2005) postulates that preparations with molecular weight in the range of 0.5 to $2.0 * 10^6$ Da may be optimal.

In conclusion, hyaluronan appears to be most useful in the treatment of acute cases with mild to moderate synovitis and capsulitis. In treatment of more severe or chronic cases, hyaluronan therapy alone does not seem adequate (Frisbie, 2006a; Caron & Genovese, 2003; Howard & McIlwraith, 1996). More research within this area is certainly needed.

Polysulfated glycosaminoglycan (PSGAG)

PSGAG suspensions for intra-articular or intra-muscular injection consist mainly of the glycosaminoglycan chondroitin sulphate which is normally found in the proteoglycans of articular cartilage. It has anti-inflammatory properties due to inhibition of MMP activity, PGE_2 synthesis and cytokine release. It has also been suggested to have a chondroprotective effect, stimulate the synoviocytial production of hyaluronan as well as promote the synthesis of proteoglycans and collagen by the chondrocytes. The precise details are not reported and controversies about the effects exist (Frisbie, 2006a; Caron & Genovese, 2003).

Improvements have been noted concerning clinical lameness, radiographic progression as well as joint morphology in cases with acute synovitis. Gaustad & Larsen (1995) found PSGAG as efficient as sodium hyaluronate in the treatment of traumatic arthritis. However, a risk of reduction in endogenous repair of cartilaginous lesions has been illuminated and therefore care should be taken when significant cartilage lesions exist (Frisbie, 2006a). In addition, there is an increased risk of infection following intra-articular administration of PSGAG (Caron, 2005) and hence PSGAG should not be administered into septic or acutely inflamed joints (Läkemedelsindustriföreningen, 2006).

Combination therapies

There has been little controlled research concerning the effects of combination therapies of osteoarthritis but it has been practiced for years in both human and equine patients. Information concerning the efficacy, however, is therefore subjective. For instance, it has been suggested that hyaluronan in the combination with glucocorticoids may help to prevent some of the potential deleterious effects of intra-articular corticosteroid administration (Howard & McIlwraith, 1996).

MATERIAL AND METHODS

The study was approved by the Ethical Committee of Animal Experiments in Uppsala, Sweden.

Group selection and sample collection

Synovial fluid was collected from a total of 10 fetlock joints (front legs) from 5 horses (Table 1). The inclusion criteria were that horses should be clinically sound (less than one degree of lameness was accepted: American Association Equine Practitioners (AAEP) classification 0-5, where five equals non-weight bearing) and, upon provocation with flexion tests, showed no more than two degrees of lameness. In addition, the horses should not have any signs of joint pathology at radiological examination.

Table 1: Horses included in the study

Horse	Race	Age (years)	Gender	Experimental fetlock joint
A	Swedish trotter	7	Gelding	Left
B	Swedish trotter	13	Mare	Right
C	Swedish warmblood	20	Mare	Left
D	Swedish trotter	10	Mare	Left
E	Swedish warmblood	10	Gelding	Left

The first day (day 0), the horses arrived and were clinically examined including palpation of the extremities and ocular inspection at walking, trotting and trotting after a one-minute flexion test. This was in all cases performed by the same veterinarian. On day one (1), samples of synovial fluid from the experimental and the opposite (control) joints were aspirated and two ml of the hyaluronan X was injected intra-articularly into the experimental joint. The horses were then kept in stall rest until the next day. On day two (2), the horses were examined and gait asymmetries were recorded at walk, trot and after flexion test. A synovial sample from the experimental joint was collected. The five following days (3-7), the horse was kept in the stall at night and in a sand paddock in daytime. Daily ocular examinations were performed and on day eight (8), the horses were evaluated for gait asymmetries and a synovial sample from the experimental joint was collected. This was followed by another two weeks with the horse kept in a paddock with daily ocular examinations followed by a clinical examination and synovial sample

collection at day 15 and day 21. On day 22, the horse was anesthetized and euthanized. Synovial fluid from both fetlock joints of the front legs was collected post mortem.

After clipping and disinfection of the skin, the synovial fluid was aspirated aseptically and transferred into sterile blood collection tubes. All synovial fluid samples were kept at 4-8 ºC for a maximum of 2 hours until centrifuged for 5 minutes (Beckmans Microfuge Lite Centrifuge; 13000 rpm; 4 ºC). The supernatant was aliquoted (150-250 µl) and stored frozen (-70ºC) until analysed. All samples were collected within a period of 4 months and analysed within 9 months after the first sample was collected.

ELISA for equine TNFα

TNFα was analysed using Equine TNFα Screening Set (Endogen, Rockford, USA) according to the manufacturer's instructions except for pre-treatment with hyaluronidase. This was done to minimize the risk that the hyaluronic acid in the sample would interfere with the binding of TNFα in the assay and therefore remove a portion of the cytokine (Billinghurst et al., 1995). All samples were analyzed at one occasion.

The 96-well plate was coated with 100 µl anti-equine antibody (3 µg/ml) per well overnight at room temperature (RT; 22-25 ºC). The next day, the plate was washed and incubated for one hour with blocking buffer.

All samples were treated with hyaluronidase by dissolving 10 mg of Hyaluronidase (Sigma) in 20 ml of Tris-Hac-Mix (SVA) to a concentration of 500 µg/ml. Then 100 µl of each synovial sample was mixed with 100 µl of the hyaluronidase buffer solution, incubated at RT for 30 minutes and 200 µl of reagent diluent was added, resulting in four times final dilution of the sample.

A standard curve (Fig. 8) covering a range of 7.8-1000 pg/ml was prepared using equine TNFα included in the kit. 100 µl of standard or sample were added to the wells in duplicates. The plate was covered with a plate sealer and incubated at RT for one hour. The plate was then washed three times with 300 µl of wash buffer per well.

The detection antibody was diluted to a concentration of 0.4 µg/ml. 100 µl of the solution was added to each well and the plate was incubated for one hour at RT. The plate was washed with wash buffer and 100 µl of Streptavidin-HRP (SA-HRP) was added to the wells and incubated for 30 minutes at RT.

Finally, the plate was washed with wash buffer before 100 µl of substrate solution was added to each well and the plate was incubated in the dark for 20 minutes at RT. The reaction was then stopped by adding 100 µl of stop solution (0.18 M sulphuric acid) to each well. The absorbance was measured in a spectrophotometer at $A_{450} - A_{550}$.

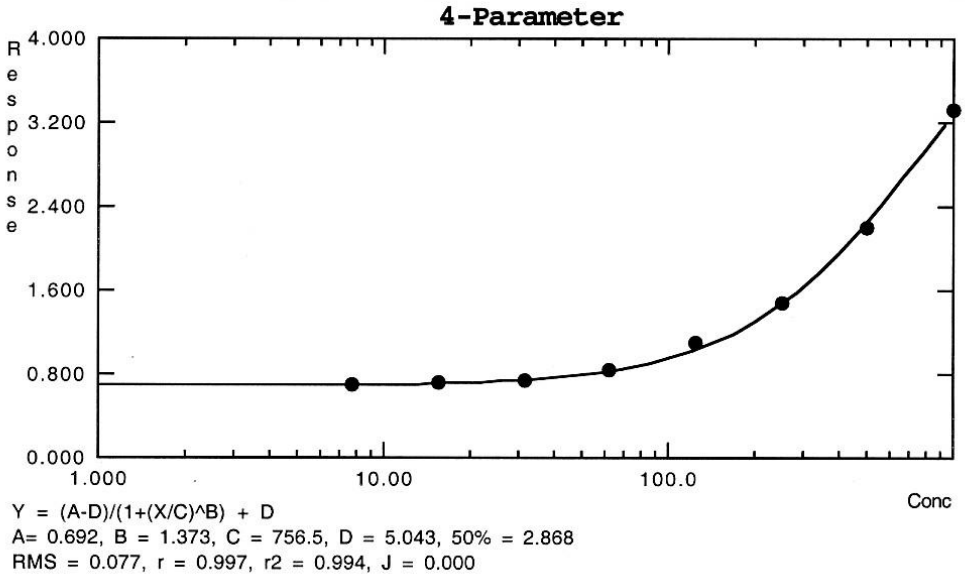

Fig 8 Absorbance vs concentration of standard.

RESULTS

Clinical response- The clinical response of the horses was summarized in another degree project (Thorell, 2007). All five horses showed increased synovial effusion the day after the injection of Hyaluronan X (day 2). In one horse (A), this disappeared and was not apparent on day 8. In horse B, the synovial effusion was still apparent day 8. In three horses (C, D, E), the increased synovial effusion was present the entire period (day 2 – day 22). Three of the horses (B, D, and E) also showed signs of increased temperature in the skin of the experimental fetlock joint (subjectively estimated). Two of the horses (B, D) showed lameness day 2; horse D showed a 0.5 degree of lameness at the trot and horse B reacted with a one degree of lameness after flexion test. At day 8, these horses were no longer lame.

The five horses in this study remained healthy throughout the study (measured as no signs of depression or increase in body temperature).

Biochemical response- The synovial fluid from the experimental joint of horse C, at days 2 and 8 presented detectable levels of TNFα. At day 15, TNFα was again non detectable. In the control joint of horse C, as well as in all the samples (from both experimental and control joints) from the other horses, TNFα was non detectable (Table 2).

*Table 2: The concentration of TNFα in the different equine samples**

Horse	Joint	Concentration of TNFα (pg/ml) Day 1	Day 2	Day 8	Day 15	Day 22
A						
	EJ	n.d.	n.d.	n.d.	n.d.	n.d.
	CJ	n.d.				n.d.
B						
	EJ	n.d.	n.d.	n.d.	n.d.	n.d.
	CJ	n.d.				n.d.
C						
	EJ	n.d.	142	210	n.d.	n.d.
	CJ	n.d.				n.d.
D						
	EJ	n.d.	n.d.	n.d.	n.d.	n.d.
	CJ	n.d.				n.d.
E						
	EJ	n.d.	n.d.	n.d.	n.d.	n.d.
	CJ	n.d.				n.d.

** EJ= experimental joint; CJ= control joint; n.d.= non detectable*

DISCUSSION

The purpose of this study was to evaluate if injection of hyaluronan X could be harmful and lead to an inflammatory reaction in the joint. This was done mainly by registering clinical signs of inflammation (covered in another degree project; Thorell, 2007) and partly by the aid of a biochemical marker. The marker chosen was the cytokine TNFα which, in several studies, has been concluded to be a good predictor of acute arthritis (Bertone et al, 2001; Jouglin et al, 2000; Billinghurst et al, 1995). The present study was a pilot project, designed to evaluate if any side effects occurred with the intra-articular administration of hyaluronan X. This was only done in a small number of horses (five) in order to minimise unnecessary pain and discomfort in a large number of horses (which will be needed in a clinical trial of hyaluronan X).

Results of this study indicate that there is an inflammatory reaction after injection of this hyaluronan suspension. Mild transient reactions are common sequelae after joint injections and the importance is not clear. One of these horses, however, reacted with lameness. This was not the same horse which presented a detectable level of TNFα in synovial fluid from the fetlock joint. Explanations for this can only be speculative. Essential is that the joint microenvironment is complex and many factors interact.

There are other methods for analyzing TNFα in synovial fluids such as bioassays. There is a possibility that a bioassay would have resulted in a higher percentage of the horses showing an increased level of TNFα following the injection. However, the immunoassay used in this study is equine specific and has a high sensitivity; hence the results with low TNFα levels are reliable. In addition, several other inflammatory mediators exist that would be suitable as biochemical markers. A complete battery of different inflammatory mediators, such as IL-1, IL-6 and PGE, would serve better to identify early inflammation. It is, however, crucial that the assays to be used are equine specific and not only evaluated in other species.

One possible explanation to the inflammatory reaction may be that the hyaluronan X, which was manufactured by an independent company, was not standardly produced. It was revealed that some of the samples of Hyaluronan X were accidentally contaminated by ethanol. Various alcohols, such as polyvinyl alcohol and ethyl alcohol, have in prior studies been shown to be irritative to the joint structures (Shoemaker, R.W. et al., 2006; Cornelissen et al., 1998). Therefore, there is a risk that the ethanol in the suspension injected, per se, might have caused the inflammatory reaction. Before any conclusions can be made of the reactions to hyaluronan X, the process of manufacturing must be properly standardised and optimised in order to avoid, for instance, contamination.

However, the results may also have been influenced by the design of the study. In order to completely evaluate the effect of hyaluronan X, there is a need for repeated sampling from a control joint as well. In this study, the opposite fetlock joint was used as a control. However, synovial fluid was only collected from this control joint at two occasions; the null sample (day 1) and the sample post mortem (day 22). The fact that the number of samples from the experimental joints and the control joints was not equal is a potential drawback; the arthrocentesis per se could be assumed to elicit an inflammatory response and hence a corresponding increase in the level of TNFα in the experimental joint. In that case, an increased level of TNFα in samples from the experimental joint could not be concluded to exclusively originate from the injected substance. However, while there are studies showing a small and limited increase in inflammatory cells following synovial aspiration, a number of studies have failed to show any effect of serial arthrocentesis on synovial TNFα (van den Boom et al, 2004; Billinghurst et al, 1995; Wagner et al, 1982). In addition, Lohmander et al (1998) found that weekly aspiration of synovial fluid was not a confounding factor in longitudinal human patient studies. They conclude that weekly injections neither have an effect on joint tissue metabolism nor induce synovitis. Also, the present study did not reveal high levels of TNFα indicating an inflammatory response to repeated arthrocentesis. Only synovial samples from one horse showed detectable TNFα levels. Therefore, this should not represent a confounding factor in the present study. However, repeated sampling of synovial fluids from experimental as well as control joints should be considered in future studies.

In addition, the control joints of the five horses did not receive any injected solution at all. Injection of any substance into control joints constitutes a potential problem since there is no obvious optimal substance. The most natural substance available should be isotonic saline solution. However, studies with saline injections have shown an effect on the joint microenvironment in both normal and diseased joints (Gaustad et al., 1999; Gaustad & Larsen, 1995). This indicates that

saline solution is not an optimal choice. Further, there are also reports describing an inflammatory response after injection of isotonic saline solution. This inflammatory response was manifested as an increased leukocyte count, a parameter which has been correlated with TNFα concentration in other studies (Bertone et al., 2001; Hawkins et al., 1993; Wagner et al., 1982)

CONCLUSION

From this study, no conclusions can be drawn about the side effects of hyaluronan X. Improvements in the manufacture of the suspension, as well as the design of the study, are necessary.

ACKNOWLEDGEMENTS

Jag vill särskilt framföra min stora tacksamhet till:

Min huvudhandledare **Stina Ekman** för all hjälp med det praktiska genomförandet och inte minst för utmärkt handledning i skrivandets konst.

Min biträdande handledare **Cecilia Ley** för enormt stöd och hjälp med provinsamlingen.

Min biträdande handledare **Maija-Leena Eloranta** på institutionen för medicinska vetenskaper på Uppsala Universitet för mycket uppskattad hjälp med ELISA. Ett stort tack också till **Anne Trönnberg** för utmärkt handledning och mycket tålamod under det praktiska genomförandet av ELISAn.

Carina Ingvast-Larsson för konstruktiv kritik angående de farmakologiska bitarna av mitt arbete.

Jim Åkerblom för idén till projektet. **Karin Roethlisberger-Holm** och **Karin Thorell** för utmärkt samarbete. **Personalen på KROJ** som tog väl hand om hästarna under studien.

Min familj, **Jesper** och **Joakim**, för att ni har kämpat med mig den här tiden. Tack för att ni ständigt påminner mig om vad som är viktigt i livet och piggar upp mig de stunder som allt bara känns jobbigt. Tack för att ni har visat tålamod när jag har visat er mina sämsta sidor. Älskar er så himla mycket!

Mina föräldrar **Evy** och **Per-Olof** samt min lillsyster **Emma** för att ni alltid finns där och ställer upp när det behövs. Tack för att ni gav mig möjligheten att bli veterinär och tack särskilt för all hjälp med studierna, hästarna och husbygget. Ni är bäst!

Sara Persson för allt stöd under den sista delen av utbildningen. Tack för sällskapet under tentapluggandet. Tack också för allt prat, alla musikupplevelser, sommaren i Vårgårda, alla luncher och fikastunder, och sist men inte minst all kunskap som du frikostigt delar med dig av. Tack för att du är en så bra vän! Utan dig hade detta aldrig gått.

Alla mina vänner i Uppsala och resten av världen som på ett eller annat sätt har bidragit till den här långa resan! Medstudenter, stallkompisar, barndomsvänner… Ingen nämnd, ingen glömd!

REFERENCES

Aviad, A.D and Houpt, J.B. (1994) The molecular weight of hyaluronan- how significant is it? J Rheumatol, 21(2), 297-301

Bertone, A.L., Palmer, J.L., and Jones, J. (2001) Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. Vet Surg, 30, 528-538

Billinghurst, R.C., Fretz, P.B., and Gordon, J.R. (1995) Induction of intra-articular tumour necrosis factor during acute inflammatory responses in equine arthritis. Equine Vet J, 27 (3), 208-216

van den Boom, R., Brama, P.A.J., Kiers, G.H., DeGroot, J., Barneveld, A., and van Weeren, P.R. (2004) The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor α activities in normal equine joints. Equine Vet J, 36 (2), 155-159.

Caron, J.P. (2003) Osteoarthritis. In: Ross, M.W. and Dyson, S.J. (Eds) *Diagnosis and Management of Lameness in the Horse*. 572-591. St. Louis: Saunders Co.

Caron, J.P. (2005) Intra-articular injections for joint disease in horses. Vet Clin Equine, 21, 559-573

Caron, J.P. and Genovese, R.L. (2003) Principles and practices of joint disease treatment. In: Ross, M.W. and Dyson, S.J. (Eds) *Diagnosis and Management of Lameness in the Horse*. 746-764. St. Louis: Saunders Co.

Cornelissen, B.P.M., Rijkenhizen, A.B.M., van den Hogen, B.M., Rutten, V.P.M.G. and Barneveld, A. (1998) Experimental model of synovitis/capsulitis in the equine metacarpophalangeal joint. AJVR, 59 (8), 978-985

Creamer, P (1997) Intra-articular corticosteroid injections in osteoarthritis: do they work and if so, how? Ann Rheum Dis 56, 634-635.

Frisbie, D.D. (2006a): Principles of treatment of joint disease. In: Auer, J.A. and Stick, J.A. (Eds) *Equine Surgery*, 3rd ed. 1055-1073. St. Louis: Saunders Elsevier Inc.

Frisbie, D.D. (2006b): Synovial joint biology and pathobiology. In: Auer, J.A. and Stick, J.A. (Eds) *Equine Surgery*, 3rd ed. 1036-1055. St. Louis: Saunders Elsevier Inc.

Gaustad, G., Dolvik, N.I., and Larsen, S. (1999) Comparison of intra-articular injection of 2 ml of 0.9% NaCl solution with rest alone for treatment of horses with traumatic arthritis. AJVR, 60(9), 1117-1121

Gaustad, G., and Larsen, S. (1995) Comparison of polysulphated glycosaminoglycan and sodium hyaluronate with placebo in treatment of traumatic arthritis in horses. Equine Vet. J, 27 (5), 356-362

Hawkins, D.L., MacKay, R.J., Gum, G.G., Colahan, P.T., and Meyer, J.C. (1993) Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6, and prostaglandin E_2 values in horses. AJVR, 54 (3), 379-386

Howard, R.D. and McIlwraith, C.W. (1996) Hyaluronan and its use in the treatment of equine joint disease. In: McIlwraith, C.W. and Trotter, W.B. (Eds) *Joint Disease in the Horse*. 257-269. Philadelphia: W.B. Saunders Co.

Jouglin, M., Robert, C., Valette, J-P, Gavard, F., Quintin-Colonna, F., and Denoix, J-M. (2000) Metalloproteinases and tumor necrosis factor-alpha activities in synovial fluids of horses: correlation with articular cartilage alterations. Vet Res, 31, 507-515

Lindholm, A., Roneus, B., Lindblad, G., Jones, B. (1996): Hyaluronan turnover in the synovial fluid in metacarpophalangeal and middle carpal joints in standardbred horses, Acta Vet Scand, 37, 147-151

Lohmander, L.S., Dahlberg, L., Eyre, D., Lark, M., Thonar, E., and Ryd, L. (1998) Longitudinal and cross-sectional variability in markers of joint metabolism in patients with knee pain and articular cartilage abnormalities. Osteoarthritis and Cartilage, 6**,** 351–361

Läkemedelsindustriföreningen (LIF). Hemsida.[online] Tillgänglig: http://www.fass.se/ [2006-12-21]

Moreland, L.W. (2003) Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action, Arthritis Res Ther, 5, 54-67

McIlwraith, C.W. (1996) General pathobiology of the joint and response to injury. In: McIlwraith, C.W. and Trotter, W.B. (Eds) *Joint Disease in the Horse*. 40-70. Philadelphia: W.B. Saunders Co.

McIlwraith, C.W. (2006) Anatomy and Physiology of Equine Joints. At: Equine Orthopaedic Center Colorado State University. Home page [online] Available from: http://www.equineortho.colostate.edu/questions/anatomyjoint.htm [2007-01-04]

Pain Relief Cushions. Homepage [online] (2006) Available from: http://www.painreliefcushions.co.uk/conditions/osteoarthritis.html [2007-01-04]

Palmer, J.L, and Bertone, A.L. (1994) Joint structure, biochemistry and biochemical disequilibrium in synovitis and equine joint disease, Equine Vet J, 26 (4), 263-277.

Philips, M.W. (1989) Clinical trial comparison of intra-articular sodium hyaluronate products in the horse. Equine Vet Sci, 9 (1), 39-40

Popot, M.-A, Bonnaire, Y., Guechot, J., and Toutain, P-L. (2004) Hyaluronan in horses: physiological production rate, plasma and synovial fluid concentrations in control conditions and following sodium hyaluronate administration, Equine Vet J, 36 (6), 482-487

Rang, H.P., Dale, M.M., Ritter, J.M., and Moore, P.K. (2003a) Local hormones, inflammation and immune reactions. In: Rang, H.P., Dale, M.M., Ritter, J.M., and Moore, P.K. (Eds) *Pharmacology.* 229-241. London: Churchill Livingstone

Rang, H.P., Dale, M.M., Ritter, J.M., and Moore, P.K. (2003b) The pituitary and adrenal cortex. In: Rang, H.P., Dale, M.M., Ritter, J.M., and Moore, P.K. (Eds) *Pharmacology.* 411-420. London: Churchill Livingstone

Ray, C.S., Robin Poole, A., and McIlwraith, C.W. (1996) Use of synovial fluid and serum markers in articular disease. In: McIlwraith, C.W. and Trotter, W.B. (Eds) *Joint Disease in the Horse*. 203-216. Philadelphia: W.B. Saunders Co.

Shoemaker, R.W., Allen, A.L., Richardsson, C.E., and Wilson, D.G. (2006) Use of intra-articular administration of ethyl alcohol for arthrodesis of the tarsometatarsal joint in healthy horses. AJVR, 67 (5), 850-857

Thorell, K. (2007) Ledinflammationer på häst och intraartikulär behandling med hyaluronsyra. Available from: http://ex-epsilon.slu.se/archive/00001384/01/EEF.pdf

Todhunter, R.J., Fubini, S.L., Vernier-Singer, M., Wootton, J., Lust, G., Freeman, K.P., and MacLeod, J.N. (1998) Acute synovitis and intra-articular methylprednisolone acetate in ponies. Osteoarthritis Cartilage, 6, 94-105

Trumble, T.N., Trotter, G.W., Thom Oxford, J.R., McIlwraith, C.W., Cammarata, S., Goodnight, J.L., Billinghurst, R.C., and Frisbie, D.D. (2001) Synovial fluid gelatinase concentrations and matrix metalloproteinases and cytokine expression in naturally occurring joint disease in horses, AJVR, 62 (9), pp 1467-1477

Wagner, A.E., McIlwraith, C.W., and Martin, G.S. (1982) Effect of intra-articular injection of orgotein and saline solution on equine synovia. AJVR, 43 (4), 594-597