BIOCHEMICAL CHARACTERIZATION OF NORMAL NAVICULAR BONE FLEXOR SURFACE CARTILAGE

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(ABSTRACT)

Cartilage tissue specimens were obtained from the flexor surface of the navicular bone and distal radiocarpal bone articular surface (controls) from 8 horses 2 to 5 years old. Water, DNA, total collagen, total glycosaminoglycans, chondroitin sulphate, and keratan sulphate contents were determined. The results from each site were compared and the differences were analyzed by paired t-test ($P < 0.05$).

Significant differences were determined between the water content of the navicular bone flexor surface cartilage (68.32± 3.46 %) and the distal radiocarpal bone articular surface cartilage (60.60± 4.09%). The total DNA content, total glycosaminoglycan content, total chondroitin sulphate content, and total keratan sulphate for the flexor surface of the navicular bone was: 524.51± 92.89 ng, 0.1533± 0.0338 mg, 0.1018± 0.0197 mg 0.0800± 0.0176 mg, and 0.0092± 0.0037 mg per mg of dry weight cartilage, respectively. The total DNA content,

total glycosaminoglycan content, total chondroitin sulphate content, and total keratan sulphate for the distal radiocarpal articular surface cartilage was: 508.80± 70.16 ng, 0.1686± 0.00838 mg, 0.0919± 0.0191, 0.0615± 0.0109 mg, and 0.0074± 0.0029 mg per mg dry weight cartilage, respectively. Not significant differences were determined between these values.

We concluded that the cartilage of the flexor surface of the navicular bone is biochemically similar to hyaline articular cartilage, but differs from previous descriptions of fibrocartilage. Further studies are needed to determine types and proportions of collagen types of the flexor surface of the normal navicular bone. These findings establish a basis of comparison to assess navicular cartilage in aging, disease, and repair.

To my parents

for their continuous encouragement and support,

to Stephane for his love.

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INTRODUCTION

Navicular disease is a chronic progressive condition that affects the navicular bone, navicular bursa, and adjacent surface of the deep digital flexor tendon. Although many aspects of the etiology and pathogenesis of navicular disease remain unclear, there is increasing evidence suggesting that the condition is a degenerative disorder similar to osteoarthritis of diarthrodial joints (Youatt, 1836; Drake, 1887; Oxspring, 1935; Doige and Hoffer, 1983; Svalastoga and Nielsen, 1983; Svalastoga and Smith, 1983; Svalastoga et al., 1983; Rooney, 1986; Pool et al., 1989; Thompson et al., 1991; Pleasant et al., 1993; Wright et al., 1998; Viitanen et al., 2001). The degenerative changes noted in navicular disease are thought to be initiated and promoted by excessive and sustained compressive forces exerted against the flexor surface of the navicular bone. Classic lesions associated with navicular disease include erosion and ulceration of the cartilage on the flexor surface of the navicular bone, osteitis and rarefaction of the flexor cortex, degeneration and cavitations of the cancellous bone, and chronic synovitis/bursitis and tearing of the fibers of the deep digital flexor tendon where it contacts the navicular bone (Hickman, 1989). It is important to note that, almost without exception, the pathologic changes associated with navicular disease are restricted to the flexor aspect of the bone (Pleasant et al., 1993). Other findings compatible with navicular disease include an increase in size and alteration of the shape of the distal border foramina and cystic changes.

However, it has been demonstrated that many of these changes can be found in clinically normal horses (Turner et al., 1986; Kaser-Hotz and Ueltschi, 1992).

Most of the information pertaining to the composition of the cartilage of the flexor surface of the navicular bone has been inferred from gross observation and from histological and histochemical studies (Svalastoga et al., 1983; Pleasant et al., 1993). In these studies, matrix depletion (decrease in proteoglycan staining) and decreased cellularity have been described in horses with navicular disease. To our knowledge, there are no reports concerning the biochemical composition of the cartilage on the flexor surface of the navicular bone in normal or diseased states.

LITERATURE REVIEW

Cartilage composition

Cartilage is a specialized form of connective tissue composed of cells (chondrocytes) interspersed in an extracellular matrix (ECM). Cartilage contains no nerves, is avascular, and alymphatic. Because cartilage is aneural, pain perception in synovial structures is dependent on nerve endings in the synovium, capsule, and subchondral bone. Because cartilage is avascular and alymphatic, nutrition and elimination of waste products are dependent on diffusion through the cartilage matrix and from the synovial fluid. Three types of cartilage have been recognized: hyaline, elastic, and fibrocartilage. The different cartilage types are distinguished on the basis of the amount of their ECM and the relative abundance of the collagenous and elastic fibers embedded in it (Fawcett, 1986).

Chondrocytes occupy .01 – 12% of the volume of cartilage, and are the only living matter in cartilage. Chondrocytes in general have a relatively low metabolic activity (Gardner, 1992). They are able to function under almost anaerobic conditions, but they are sensitive to toxic influences and are unable to regenerate after major injury. Cartilage loading stimulates diffusion of chondrocyte nutrients and waste products through the cartilage matrix and is therefore essential to cartilage nutrition. The chondrocytes have the capacity to synthesize as well as degrade all components of the extracellular matrix. The metabolism of

chondrocytes is influenced by a variety of biomechanical and biochemical stimuli, and under normal circumstances, the chondrocytes produce precisely regulated amounts of proteases and protease inhibitors to induce normal turnover of the ECM components. The rate of turnover of the matrix components is variable; the proteoglycan pool is typically renewed at a relatively rapid rate, whereas the rate of collagen fiber turnover is extremely slow (Todhunter, 1996).

The biochemical composition of the ECM varies with cartilage type, from individual to individual, from site to site, and with age (Dijkgraaf et al., 1995). The ECM of all cartilage types is composed of water, protein fibers (collagen, elastin), proteoglycans, and small amounts of lipid and inorganic components. The biochemical composition of the cartilage ECM determines the biomechanical characteristics of the tissue, such as strength, resilience, and elasticity.

Collagen is the major constituent of cartilage ECM (40 – 60% of dry weight). Collagen molecules are composed of a triple helix of polypeptide chains, called α chains. The collagen molecules align with 25% overlap to form fibrils. The collagen fibrils in the ECM are organized in sheets and bundles, creating a network. This collagen network is kept together by its basketweave structure, cross-links, and anchoring proteins. The collagen network provides cartilage with its tensile strength and shape, and counteracts the swelling pressure of the highly hydrophilic proteoglycans.

Type II collagen is the most abundant protein fiber in hyaline cartilage. Type II collagen is composed of three identical polypeptide chains, called α_1 (II). Type I collagen is the most abundant protein fiber in fibrocartilage. Type I collagen tends to form thicker fibrils than type II collagen and differs from type II collagen in that one of the three-polypeptide chains, called α_2 (I), is different from the other two identical chains called α_1 (I). Minor collagens are present in relatively small amounts in cartilage, and their roles in its structure and function have yet to be fully defined. Minor collagens include types IX, XI, XII, and XIV. Elastic fibers are found in elastic cartilage and are composed of a protein with high elastic recoil called elastin. Elastin is rich in glycine and proline, like collagen, but differs in that it contains almost no hydroxylated amino acids.

Proteoglycans constitute 20 – 40 % of the dry weight of hyaline cartilage. The proteoglycan content of fibrocartilage has not been reported, but has been suggested to be smaller than that of hyaline cartilage (De Bont, 1985; Dijkgraaf et al., 1995). Proteoglycans are in general intertwined throughout the collagen network and are not only mechanically, but also chemically entangled in this network. Proteoglycans are highly hydrophilic macromolecules with a high water–binding capacity. They are only constrained from full expansion by the tension of the collagen network. The proteoglycans in conjunction with the collagen network provide cartilage with its resilience, elasticity, and shear strength.

Proteoglycans are complex macromolecules, consisting of a core protein with many glycosaminoglycan (GAG) side chains of varying composition and chain length, linked with hyaluronic acid by a link protein. The glycosaminoglycan side chains in cartilage are chondroitin sulphate, keratan sulphate. About 75 percent of the proteoglycans exist in aggregates. This arrangement may help protect the proteoglycans from enzymatic degradation. The remaining proteoglycans are attached to collagen and appear to give the fibrils increased mechanical stability. The glycosaminoglycan side chains are the highly charged polyanionic parts of the proteoglycan molecules. They are responsible for the hydrodynamic force of the proteoglycans. The strong tendency of the proteoglycans to imbibe water creates a swelling pressure within the cartilage, which forms the basis of the elastic resistance to compression in the cartilage.

Non-collagenous, non-proteoglycan glycoproteins constitute a small but significant portion of the cartilage and are thought to play a role in adhesion of the chondrocytes to the ECM.

Hyaline cartilage provides the nearly frictionless, wear-resistant bearing surface of most joints. Normal equine hyaline cartilage contains (by dry weight) approximately 50% collagen (85-90 % of which is type II), 35% proteoglycans, 10% glycoproteins, 3% mineral and 1% lipid; it also contains 1 to 12%

chondrocytes (by volume). The water content of equine hyaline cartilage is approximately 70% (Vachon et al., 1990; Todhunter, 1996). The major glycosaminoglycans in adult hyaline cartilage are chondroitin-4 sulphate, chondroitin-6 sulphate, keratan sulphate, and hyaluronic acid. The biochemical composition of the extracellular matrix of hyaline cartilage varies from individual to individual, between joints in the same individual, and between sites in the same joint (Dijkgraaf et al., 1995). Different joints and areas within a joint are subjected to different types of loading, subjecting each to different biomechanical challenges (Brama et al., 2001). Cartilage adapts itself to the conditions to which it is regularly subjected; by increasing proteoglycans levels in areas of increased stress, thereby increasing the stiffness of the cartilage compared to less loaded areas (Swann and Seedholm, 1993).

Cartilage-on-cartilage lubrication uses boundary lubrication and hydrostatic lubrication. Boundary lubrication operates at low loads and requires a glycoprotein lubrication fraction. At high loads the joint is lubricated by hydrostatic or squeeze-film lubrication from the cartilage it self (Radin and Paul, 1972).

Fibrocartilage is found in areas subjected to great stress or weight bearing such as menisci. Fibrocartilage is a dull white tissue, and is reportedly stronger and less deformable than hyaline cartilage. It has been described as a transitional form of tissue between cartilage and dense connective tissue, having

characteristics similar to both tissues (Leeson et al., 1988). The fibroblast like chondrocytes of fibrocartilage synthesize matrix containing principally type I collagen, and relatively small amounts of proteoglycans (Gardner, 1992). Dijkgraaf et al. (1995), suggested that the water content of fibrocartilage is less than that of hyaline cartilage. Furthermore the keratan sulphate content is much smaller in fibrocartilage than in hyaline cartilage, whereas dermatan sulphate content is more prevalent (Dijkgraaf et al., 1995).

Elastic cartilage is very similar to hyaline cartilage except that in addition to type II collagen fibers, it contains an abundant network (as much as 20% of the dry weight of the tissue) of fine elastic fibers (Junqueira et al., 1992). The elastic fibers of the ECM make this type of cartilage highly plastic (Leeson et al., 1988). The tissues of the epiglottis and external ear exemplify elastic cartilage.

Various methods have been used to provide accurate biochemical profiles of cartilage. Concentration of DNA is used to estimate the number of chondrocytes in a given volume of tissue (Young-Jo et al., 1988; Richardson and Clark, 1991). Total collagen content can be estimated on the basis of hydroxyproline content (Reddy and Enwemeka, 1996). A modified form of dimethylmethylene blue assay in conjunction with specific polysaccharidases can be used to determine the total glycosaminoglycans and type (chondroitin sulphate and keratan sulphate) (Farndale et al., 1986; Palmer et al., 1995).

Osteoarthritis in horses

Etiopathogenesis:

Osteoarthritis is a disorder of synovial joints characterized by degeneration and loss of articular cartilage. (Kidd et al., 2001). It is considered the most economically important musculoskeletal disease affecting the horse (McIlwraith 1996; Kidd et al., 2001). Economic losses may occur in the form of expensive treatments and loss of time in training and competition, as well as due to the early termination of athletic careers. Just as important, affected horses can suffer meaningful pain and discomfort.

The normal turnover of the ECM of articular cartilage is regulated by the chondrocytes under the control and influence of mechanical stimuli and cytokines. In osteoarthritis, there is a disruption of the homeostatic state of the cartilage and catabolic processes dominate. Extracellular matrix degradation is central to the development of osteoarthritis and occurs either by direct damage or due to degrading enzymes released into the synovial fluid.

In the horse, trauma due to acute or repetitive overload is the most identifiable cause of osteoarthritis (Kidd et al., 2001). The primary injury may be to the cartilage (i.e., cartilage tear/fragmentation), the synovium/joint capsule (i.e., synovitis, ligament injury), the subchondral bone (i.e., fracture, sclerosis) or a

combination of these structures. The structures not involved initially often become involved secondarily and contribute to a pathologic sequence that continues in a cyclic manner unless arrested.

In response to acute or chronic injury to a joint in horses, the initial changes are often characterized by synovitis. As a result of the inflammatory response, leukocytes, degradative enzymes, and cytokines enter the synovial fluid from the synovium and cartilage. The synovial fluid becomes less viscous and the transit path for cartilage nutrition is disrupted. There is often a loss of chondrocytes, and the chondrocytes that remain may respond by releasing inflammatory mediators and degradative enzymes (Todhunter, 1996). The extracellular matrix undergoes a variety of changes. The water content of the cartilage increases, possibly as a result of increased water bonding to collagen or damaged collagen failing to oppose the swelling pressure of the proteoglycans. There is a loss of orientation of the collagen fibers near the cartilage surface with the fibers becoming more separated than usual. The mechanical structure of the cartilage becomes weakened, predisposing to fibrillation and cleft formation.

The increased water content of water of the cartilage allows greater extraction of proteoglycans. Proteoglycan loss occurs as a result of enzymatic degradation (Kidd et al., 2001). The chondrocytes may respond by increasing their production of proteoglycans, but the new proteoglycans often have lower molecular weights,

have an altered glycosaminoglycan structure, and do not readily form aggregates (McDevitt et al., 1977).

Once this pathologic sequence has begun it continues in a cyclic manner unless arrested by removing the inciting cause(s). The long-term outcome is determined by the chondrocytes ability to synthesize sufficient matrix components and to restore the normal joint environment.

Diagnosis:

There is an inconsistent correlation between the clinical signs of osteoarthritis and the degree of morphological change present. Much depends on the specific joint involved and the type and level of activity of the horse. Common physical findings in horses with osteoarthritis include joint effusion, decreased range of motion, and pain on flexion of the involved joint(s). Intra-articular anesthesia is often used to confirm a specific joint as the cause of lameness.

Radiography is often used in the evaluation of horses with suspected osteoarthritis. Radiographic changes consistently associated with osteoarthritis include loss of joint space, osteophytes, subchondral bone lysis, and soft tissue swelling. It is important to note that these changes are only seen in cases with advanced disease. In early osteoarthritis, there are usually no radiographic changes. Subchondral bone sclerosis has been suggested to be consistently

associated with overlying cartilage damage, but this association has not yet been confirmed. Radiographic changes such as enthesophytes, and dystrophic calcifications of capsular, ligamentous, or tendinous attachments may be evident without significant changes within the joint. In addition, osteophytes are nonspecific responses to articular disease. They can develop in horses without causing clinical signs or necessarily progressing to clinically apparent disease (May, 1996).

The “gold standard” for evaluation of joint disease in horses at this time is diagnostic arthroscopy. Arthroscopy allows direct visualization of cartilage, synovium, and intraarticular ligaments. However, the cost of general anesthesia and surgery prevent diagnostic arthroscopy from being used for evaluation of osteoarthritis on a routine basis.

Cost and availability also limit the application of diagnostic imaging modalities such as computed tomography and magnetic resonance imaging (which provide superior soft tissue resolution to conventional radiography) for horses. Advances in system designs should eventually bring these modalities into routine clinical use.

Treatment:

Treatment of osteoarthritis should be directed towards diminishing the inflammatory response in both the synovium and cartilage, halting progressive cartilage degradation, restoring nutrition, normal joint environment, and alleviating pain. The most important therapy for any injured joint is probably rest or modification of activity. Continued, unrestricted use of an inflamed joint containing cartilage that is mechanically inferior due to matrix loss is likely to lead to structural failure of the cartilage and its eventual loss. Rest or modification of activity may allow inflammation to subside, and the matrix of the cartilage to replenish before structural integrity is compromised.

A wide variety of preparations are available for treatment of joint disease. The medications most commonly used for the treatment of equine osteoarthritis are non-steroidal anti-inflammatory drugs, intra-articular corticosteroids, hyaluronic acid, polysulphated glycosaminoglycan, and oral glucosamine and chondroitin sulphate.

It has been accepted for some time that prostaglandins (PG), particularly PGE, are associated with pain production, synovial membrane inflammation, and cartilage matrix depletion (Caron, 1999). Non-steroidal anti-inflammatory drugs (NSAIDs) reduce pain and inflammation by inhibiting the enzyme cyclooxygenase and the subsequent production of prostaglandins.

Phenylbutazone is the most widely used NSAID for the treatment of joint disease in the horse (Turner, 1989). It is often used as a first line of treatment with minor joint injuries due to its ease of administration (oral and intravenous preparations). It is also used commonly in cases of chronic joint disease, predominately for the management of pain. Other NSAIDs used in the horse include aspirin, flunixen meglumine, ketoprofen, naproxen, and carprofen.

Among the medications available for the treatment of osteoarthritis, corticosteroids are the most powerful with respect to their anti-inflammatory effect. They inhibit inflammatory processes at virtually all levels, including capillary dilation, margination, migration, and accumulation of inflammatory cells, liberation of enzymes and inflammatory mediators, and the production of prostaglandins and cytokines when injected directly into affected joints (Gray et al., 1981; McIlwraith, 1996; Caron, 1999). Unfortunately, corticosteroids have also been shown to have negative effects on chondrocyte metabolism. Specifically, at high concentrations, corticosteroids inhibit proteoglycan synthesis and unfavorably influence the structural organization of cartilage collagens (Oegema and Behrens, 1981; Chunekamrai et al., 1989; Jolly et al., 1995; Todhunter et al., 1996). Concern over these observations has lead to the use of doses lower than what were previously used. Recent studies have supported this practice by demonstrating that at low doses, corticosteroids are capable of inhibiting the process of cartilage degradation by inhibitory effects on the

synthesis of degradative enzymes and cytokines, without marked effects on chondrocytes (Williams and Brandt, 1985; Pelletier and Martel-Pelletier, 1989; DiBattista et al., 1991; Pelletier and Martel-Pelletier, 1991; Amano et al., 1993; Pelletier et al., 1995; Caron et al., 1996). Corticosteroids commonly used intra-articularly in horses include triamcinolone acetonide, methylprednisolone acetate, and betamethasone.

Hyaluronic acid (HA) is a linear polydisacharide and polyionic nonsulphated glycosaminoglycan. It is an integral component of both synovial fluid and articular cartilage in synovial joints. Synovial fluid HA is synthesized by the synovioctes, and serves as the principal lubricant of the synovial soft tissues (Todhunter, 1996). The HA that is incorporated in the ECM of cartilage is synthesized by the chondrocytes, and plays a major role in the formation of proteoglycan aggregates. Beneficial effects after intra-articular administration of HA have been reported in a number of equine studies (Rydell et al., 1970; Swanstrom, 1978; Rose, 1979; Vernon, 1983; Cannon, 1985; Ruth and Swites, 1985; Galley, 1986; Phillips, 1986; Phillips, 1989). The mechanism through which beneficial effects have been achieved is uncertain. The therapeutic effects may be the result of replacement of depleted or depolymerized endogenous HA (McIlwraith, 1986), or anti-inflammatory effects such as an inhibition of chemotaxis of granulocytes, macrophages, and migration of lymphocytes, as well as reduction of phagocytosis by granulocytes and macrophages (Balazs and Darzynkiewiez,

1973; Brandt, 1974; Forrester and Balazs, 1980; Treadway et al., 1981; Pisko et al., 1983; Partsch et al. 1989; Ghosh, 1993; McIlwraith, 1996). A form of HA for intravenous use is also available. The precise mechanism or mechanisms of action of this preparation is/are also uncertain, but beneficial clinical and biochemical effects have been experimentally proven (Kawcak et al., 1997).

Polysulphated glycosaminoglycan (PSGAG) is a semisynthetic preparation from bovine trachea composed principally of chondroitin sulphate. PSGAG has been shown to inhibit the effects of various lysosomal enzymes associated with cartilage degradation, and inhibit PGE2 synthesis and cytokine release (Kruze et al, 1976; Stancikova et al., 1977; Baici et al., 1980; Dietmar, 1983; May et al., 1988; Altman et al., 1989; Theiler et al., 1994). In addition to these anti-inflammatory and antidegradative effects, PSGAG has been shown to stimulate the synthesis of hyaluronic acid by synoviocytes, and proteoglycans and collagen by chondrocytes (Nishikawa et al., 1985; Smith and Ghosh, 1986; Glade, 1990). PSGAG can be administered intra-articularly or intramuscularly, but there is an elevated risk of infection accompanying intra-articular administration (Gustafson et al., 1989).

A number of oral supplements (nutraceuticals) have been developed to assist in the management of osteoarthritis and joint pain in a variety of species, including the horse, and there are now several studies demonstrating their beneficial

effects (Crolle and D'Este, 1980; Drovanti et al., 1980; Lopez, 1982; Morreale et al., 1996; Hanson et al., 1997a,b,c; Basleer et al., 1998; Bucsi and Poor, 1998; Das et al.,1998; Dorna and Guerrero, 1998; McCarty, 1998; Qui et al., 1998; Uebelhart et al., 1998; Verbruggen et al., 1998; Leffler et al.,1999; Platt, 2001). The composition of these formulations varies, but they usually contain either chondroitin sulphate or glucosamine, or a combination of chondroitin sulphate and glucosamine. Most researchers consider that providing a combination of chondroitin sulphate and glucosamine has greater beneficial effects than either compound alone (Platt, 2001).

Navicular disease

Navicular disease has been recognized as a common cause of forelimb lameness in horses for more than 200 years. In a one study, it was estimated to be the cause of one-third of the chronic lamenesses in horses (Colles, 1982). Signs attributed to pain from the palmar half of the foot characterize the disease. Horses of various breeds and uses may be affected, with some becoming sufficiently lame that they are no longer useful.

Anatomy:

The navicular bone is one of three sesamoid bones in the distal limb of horses. The navicular bone is canoe-shaped and its long axis is oriented horizontally. It is situated palmar (plantar) to the junction of the second and third phalanges and

forms the palmar (plantar) portion of the distal interphalangeal joint. It has two surfaces (articular and flexor), two borders (proximal and distal), and two extremities (medial and lateral) (Figure 1).

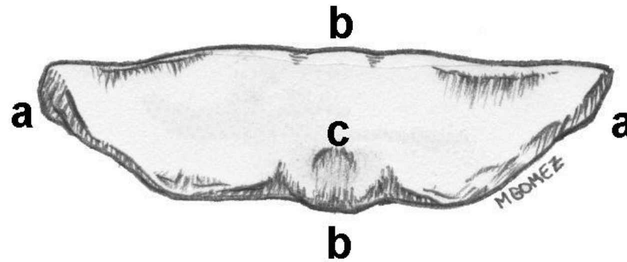

Figure 1: Diagram of the flexor surface of the navicular bone. (a) Extremities, (b) borders, and (c) flexor surface of the navicular bone.

The articular surface faces proximal and dorsal, is covered by hyaline cartilage, and articulates with the distal end of the second phalanx. The flexor surface is directed distally and palmarly. This surface has a prominent sagittal ridge and is covered by cartilage (suggested as being fibrocartilage) (Figure 2). The flexor surface of the navicular bone apposes the deep digital flexor tendon as it passes to its insertion on the third phalanx. The distal border of the navicular bone has a small facet covered by hyaline cartilage for articulation with the third phalanx. Several foramina lie in a groove palmar to this facet. These foramina are lined with synovial membrane from the distal interphalangeal joint and penetrating vessels (Poulos and Smith, 1988). Palmar to these foramina is a prominence

where the distal sesamoidian impar ligament attaches. The extremities of the navicular bone are blunt pointed.

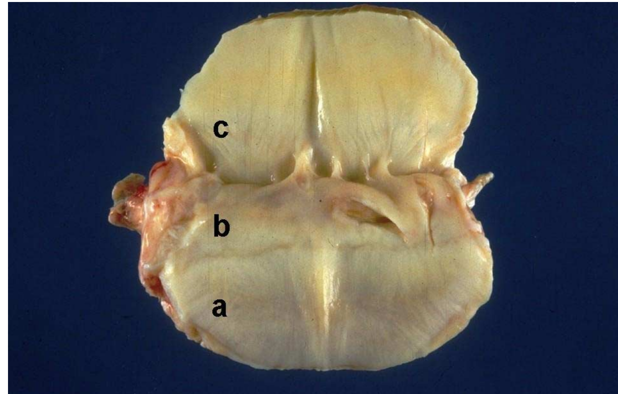

Figure 2: Normal flexor surface of the navicular bone of a horse. (a) Flexor surface navicular bone, (b) dorsal cul-de-sac navicular bursa, and (c) apposing surface of the deep digital flexor tendon (flexor tendon reflected dorsally).

The navicular bursa is located between the flexor surface of the navicular bone and the deep digital flexor tendon (Figure 3). It extends from about 1.5 cm proximal to the navicular bone distally to the insertion of the deep digital flexor tendon on the third phalanx. The navicular bursa has numerous long villi in its proximal and distal cul-de-sacs and is histologically similar to other synovial membranes of horses (Hoffer, 1982). The navicular bursa and the distal

interphalangeal joint do not communicate directly in horses (Calislar and St. Claire, 1969; Gibson et al., 1990; Gough et al., 2002).

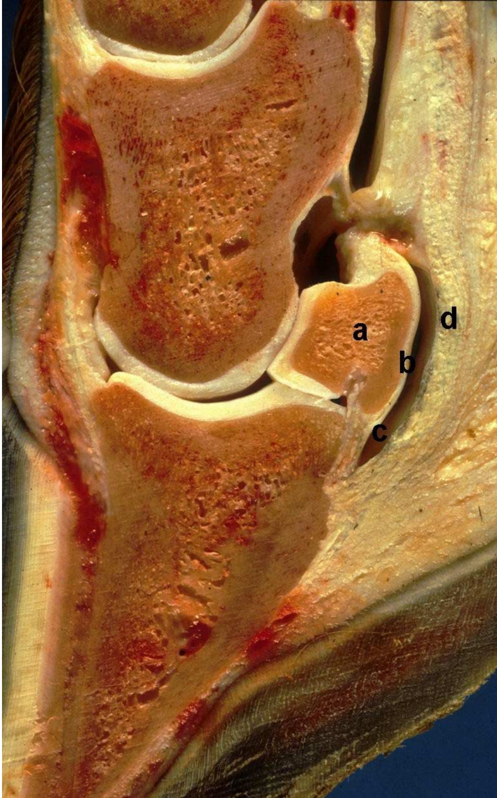

Figure 3: Sagittal section of the distal interphalangeal joint in a horse. (a) navicular bone, (b) flexor surface of the navicular bone, (c) navicular bursa, and (d) deep digital flexor tendon.

Three ligaments support the navicular bone. Paired collateral sesamoidian ligaments arise from the distal end of the first phalanx and attach to the proximal

border and extremities of the navicular bone. Distally, the distal sesamoidian impar ligament stabilizes the navicular bone. This ligament attaches the distal border of the navicular bone to the palmaroproximal surface of the third phalanx.

The navicular bone's blood supply originates from two anastomoses between the lateral and medial digital arteries. Branches from the conjoined palmar arteries of the second phalanx supply an anastomotic network that provides several small arteries to the foramina along the bone's proximal border. Distally, connecting branches between the digital arteries give off several small arteries that travel through the distal sesamoidian impar ligament and enter the foramina in the distal border of the navicular bone. In adult horses, anastomoses occur between the proximal and distal branches within the navicular bone. The distal arteries supply the majority of the substance of the navicular bone (Colles and Hickman, 1977; Hertsch and Dammer, 1988; Rijkenhuizen et al., 1989).

The digital nerves innervate the navicular region. Nerve fibers travel through the collateral sesamoidian ligaments and distal sesamoidian impar ligament and enter the navicular bone along with the nutrient arteries through the proximal and distal foramina. Sensory nerve fibers have also been demonstrated within the synovial linings of the navicular ligaments and navicular bursa (Bowker et al., 1994).

Development:

The navicular bone is formed from a single center of ossification (Rijkenhuizen et al., 1989: Hoffer et al., 1989). At 80-90 days of gestation the navicular bone consists of a condensation of mesenchymal cells and intercellular matrix, giving the tissue a precartilaginous appearance. At 100-120 days of gestation, the navicular bone consists of well-differentiated hyaline cartilage covered by a zone of tangentially arranged fibrocytes. The navicular bursa is well developed and exhibits complete cavitation. Differentiation of the navicular ligaments is apparent. At 330 days of gestation, the navicular bone is partially ossified centrally. Bone trabeculae within this center of ossification are randomly oriented. From the age of one to two days of birth, the trabeculae begin to arrange in a dorsopalmar orientation, perpendicular to the flexor and articular cartilage surfaces. Ossification of the navicular bone is usually complete by 3 to 4 months of age. The flexor surface of the navicular bone in the fetus and adult is covered with cartilage. A similar cartilage is present on the opposing surface of the deep digital flexor tendon (Hoffer et al., 1989). At day 100 of gestation, the flexor surface of the navicular bone is white, smooth, and glossy (Rijkenhuizen et al., 1989). After 125 days of gestation, three layers (superficial, intermediate and basal) are recognizable in the flexor surface cartilage (Rijkenhuizen et al., 1989). This cartilage is well supplied with arteries and veins during the gestational period that regress and completely disappear a few weeks after birth (Rooney, 1985). Rijkenhuizen et al. (1989) described thinning of the flexor surface cartilage

at two months of age. The articular cartilage is smooth, white, and bluish-white independent of the fetal age, and four layers are recognized from day 240 of gestation (Rijkenhuizen et al., 1989).

Function:

The proposed function of the navicular bone is to provide a constant angle of insertion for the deep digital flexor tendon onto the third phalanx, thereby improving its mechanical advantage (Rooney, 1967). The navicular bone also increases the functional area of the articular surface of the distal interphalangeal joint, and probably helps dissipate the concussive forces on the joint (Ratzlaff and White, 1989).

Biomechanical Considerations:

At least three forces act on the navicular bone: 1) compression forces from the second phalanx, 2) compression forces from the deep digital flexor tendon, and 3) tension forces from the navicular ligaments. The magnitude and effect of these forces are probably related the horse weight, use, and conformation (Pleasant and Crisman, 1997). Factors such as excessive body weight, small foot for body size, upright conformation, hoof imbalances, and work on hard ground are proposed to increase the forces acting on the navicular bone. The conditions of a high load and low motion joint are present in the navicular bursa where the

deep digital flexor tendon bends around the flexor cortex of the navicular bone to insert on the distal phalanx (Pool, 1995).

Hoof conformation probably affects the forces acting on the navicular bone more than any other factor. In clinician experience, a high pastern angle, low hoof angle (broken-back hoof pastern) conformation is common in horses with navicular disease. In horses with this type of conformation, all three forces acting on the navicular bone are likely increased. This low hoof angle, increased pastern angle conformation increases the tension on the deep digital flexor tendon, which is directly converted to increased compressive forces on the navicular bone (Lochner et al., 1980; Willeman et al., 1999). The pastern angle is elevated (Bushe et al., 1987) which probably increases the compressive forces on the navicular bone from the second phalanx and increases the tension forces on the navicular ligaments. Hoof break-over is also prolonged (Clayton, 1987) and probably further increases the tension on the deep digital flexor tendon and navicular ligaments. In addition, this type of conformation shifts the center of the weight distribution palmarly, increasing the load on the palmar aspect of the foot (Barrey, 1990). Underrun heels also occur commonly in horses with navicular disease (Turner, 1986). This conformation puts the heels further forward the foot than is ideal and results in increased concussion to the structures in the palmer portion of the foot.

The peak compressive force of the deep digital tendon on the navicular bone in a horse walking at 1.2 m/s is approximately 0.67 times the body weight and occurs at about 70-75 % of the duration of the stance (Schryver et al., 1978). At a slow trot (2.9 m/s), the peak force is 0.77 times the body weight and occurs at 65-70 % of the stance duration (Schryver et al., 1978). The peak forces at a gallop can reach 1.5 times the values at a trot (Wilson et al., 2001). Wilson et al. (2001) reported that the peak stress and stress rate experienced by horses with navicular disease meet previously established criteria for causing cartilage damage (Carter, 1984).

Etiopathogenesis:

Navicular disease is a chronic progressive condition that affects the navicular bone, navicular bursa, and adjacent surface of the deep digital flexor tendon (Figure 4). Although many aspects of the etiology and pathogenesis of navicular disease remain unclear, abundant information suggests that the condition is a degenerative disorder similar to osteoarthritis of diarthrodial joints (Youatt, 1836; Drake,1887; Oxspring, 1935; Doige and Hoffer,1983; Svalastoga and Nielsen, 1983; Svalastoga and Smith,1983; Svalastoga et al.,1983; Rooney, 1986; Pool et al.,1989; Thompson et al., 1991; Pleasant et al., 1993; Wright et al., 1998; Viitanen et al., 2001). It is important to note that, almost without exception, the pathologic changes associated with navicular disease are restricted to the flexor aspect of the bone. The distal interphalangeal joint surface of the navicular bone

is rarely affected. Other findings compatible with navicular disease include an increase in size and alteration of the shape of the distal border foramina and cystic changes. However, it has been demonstrated that many of these changes can be found in clinically normal horses (Turner et al., 1986; Kaser-Hotz and Ueltschi, 1992).

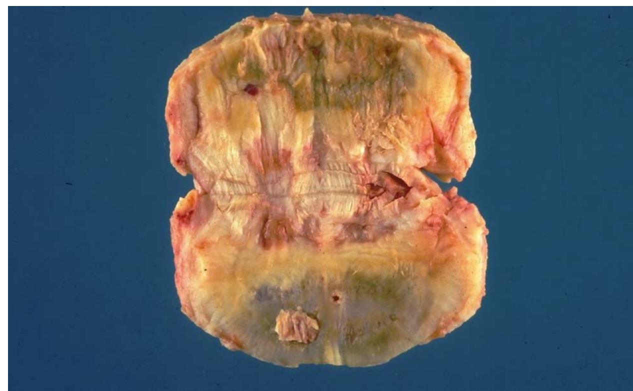

Figure 4: Flexor surface of the navicular bone of a horse with navicular disease (severe cartilage loss and tendon adhesion).

The navicular bursa is similar to a synovial joint except that the apposing surfaces are composed of the flexor surface cartilage and the deep digital flexor tendon rather than hyaline cartilage. Histologic-histochemical studies of the synovial membrane of the navicular bursa (Svalastoga and Nielsen, 1983) and the flexor surface cartilage (Svalastoga et al., 1983; Pleasant et al, 1993; Wright et al., 1998) from horses with navicular disease have demonstrated pathologic

changes similar to those seen in the synovial membrane and hyaline cartilage in osteoarthritic joints. As well, synovial fluid analyses from horses with navicular disease demonstrated elevated matrix metalloproteinases 2 and-9 enzyme activities and decreased GAG levels (Viitanen et al., 2001). The subchondral thickening and sclerosis of the flexor cortex, and fibrosis of the marrow spaces beneath the flexor cortex of horses with navicular disease are essentially the same changes that occur in horses with osteoarthritis of the proximal interphalangeal, distal intertarsal, and tarsometatarsal joints (Pool et al., 1989). Increased navicular bone intraosseous pressure has also been demonstrated in horses with navicular disease (Svalastoga and Smith, 1983; Pleasant et al, 1993). Intraosseous hypertension is a common finding in humans with osteoarthritis and is a reflection of intraosseous stasis and impaired venous drainage from the bone marrow (Arnoldi et al., 1972; Arnoldi et al., 1975; Kofoed, 1986; Kiaer et al., 1989). Increased intraosseous pressures have also been demonstrated in humans with joint pain, but without any radiographic signs of osteoarthritis (Arnoldi et al., 1975; Lemperg and Arnoldi, 1978). Intraosseous hypertension presumably causes pain by stretching the nerve endings associated with the walls of trabecular bone and dilated sinusoids and venules.

Given the similarities between navicular disease and osteoarthritis, the cause of navicular disease is most likely mechanical in nature. Navicular disease probably develops in either of two settings: 1) the horse's distal limb conformation is

normal, but abnormal loads are applied to the navicular region which cause the tissues to fail, or 2) the applied loads are physiologically reasonable, but the horses conformation causes abnormal loading of the navicular region. In either situation, the underlying concept is that there is an imbalance between the load applied and the tissues capacity to withstand that load. If the tissue damage is excessive or repetitive, navicular disease will result. Abnormal loads could be caused by overuse (work at a rate which does not allow adequate tissue repair/remodeling), work on hard ground, and even erratic use (acute bouts of overload on tissues not adapted to that use). Examples of inferior conformation would include upright pasterns, broken-back hoof pastern axes, and underrun heels. The pain experienced by horses with navicular disease could presumably come from inflammation of the navicular bursa, inflammation of the navicular ligaments, deep digital flexor tendon tendonitis, and increased navicular bone intraosseous pressure. It is likely that different horses have different degrees of pain originating from each source.

Diagnosis

Horses with navicular disease usually have a history of an insidious onset of lameness. Affected horses are usually bilaterally lame, although the lameness may be predominant in one limb. Lameness is characterized by a short, choppy gait and is usually exacerbated by work in tight circles or on hard ground. In

advanced cases, horses may point the affected limb or limbs at rest, and one or both feet may become smaller and more upright.

Various techniques are used to help diagnose navicular disease. Affected horses usually respond with pain to hoof tester pressure over the navicular region (middle third of the frog). As well, some horses with navicular disease will respond positively to distal limb flexion tests. Diagnostic anesthetic techniques are important in confirming the navicular region as the source of lameness. Affected horses usually demonstrate almost total resolution of lameness following anesthesia of the palmar digital nerves, anesthesia of the navicular bursa, and anesthesia of the distal interphalangeal joint.

Radiographic evaluation of the navicular bone is commonly performed to help diagnose navicular disease. Radiographic findings that have been considered compatible with navicular disease include an increase in size and alteration of the shape of the distal border foramina, lysis of the flexor cortex, remodeling of the proximal or distal borders of the navicular bone (enthesophytes), and loss of corticomedullary distinction. It has been demonstrated, however, that many of these changes can be found in clinically normal horses (Turner, 1982; Turner, 1989). In particular, the significance of distal border foramina changes has recently been questioned. It is also important to realize that the absence of radiographic changes does not rule out a diagnosis of navicular pain.

Scintigraphy (nuclear imaging, "bone scan") is also used to help diagnose navicular disease occasionally. Scintigraphy is probably most useful in cases where radiographic changes are equivocal or have not yet developed.

Treatment

Success or failure of treatment of navicular disease is dependent on several factors including when therapeutic intervention is initiated, the horse's conformation, and the expectations for the horse. In all likelihood, a "cure" or complete resolution of the disease is improbable unless treatment is initiated early. Therefore, treatment regimes are often directed toward pain management and prolonging the usefulness of the horse. In general, treatments that reduce stress on the navicular bone, improve navicular bone microcirculation, and retard degenerative processes in the navicular bursa are indicated.

Rest or modification of activity is an important but often overlooked component of treatment for navicular disease. Continued trauma to injured tissues in the navicular region (eg. synovium, cartilage, and bone) will ultimately compromise their structural integrity. Rest or modification of activity is important to allow soft tissue inflammation to subside and appropriate bone remodeling to occur. The amount of rest required will vary with each patient and may range from a few weeks to several months (Crisman and Pleasant, 1997). Treatment during the rest period includes correcting hoof imbalances and initiating any medical

therapies are to be employed they should be instituted at this time. When exercise is resumed, it should be done gradually and on a consistent basis. In many instances, the exercise schedule will need to be adjusted to reduce the amount and level of work in order to keep the horse useful (Crisman and Pleasant, 1997).

Corrective trimming and shoeing are the foundation of navicular disease treatment. The goal of corrective trimming and shoeing is to reduce abnormal biomechanical forces on the navicular region. It is recommended to trim the hooves to establish matched hoof-pastern axes (the slope of the dorsal surface of the hoof wall should be parallel to the angle of the pastern). In horses with very low heels, wedge pads or wedge-heel shoes may be required to achieve a parallel axis and to support the heels. Shoes should be set full to encourage hoof expansion and long to provide palmar support. Nails should be placed only in the dorsal portion of the hoof so as not to inhibit hoof wall expansion. The toe of the shoes should be rolled or rockered to ease breakover. Wide-web or aluminum shoes may help reduce concussive forces. In reality, this type of trimming and shoeing is "correct" rather than "corrective", meaning that all horses should be trimmed and shod in this manner (Pleasant, 2002). Proper hoof care from the start might reduce the incidence of navicular disease (Crisman and Pleasant, 1997).

Nonsteroidal anti-inflammatory drugs are frequently used in the management of navicular disease. NSAIDs have been recommended during the initial treatment period to reduce inflammation and pain. In some cases, NSAIDs have to be used intermittently or even chronically to keep horses usable (Turner, 1989). Dosage rates for NSAIDs vary. If used chronically, the lowest effective dose should be used in order to avoid toxicosis. Phenylbutazone is the most commonly used NSAID in the treatment of navicular disease (Turner, 1989).

Following several reports in the late 1970's describing thrombosis of the distal navicular arteries in horses with navicular disease, warfarin therapy became popular. Although current knowledge indicates that thrombosis is not a major feature of navicular disease, favorable results using warfarin reported with 58 to 75 per cent of the horses been sound for up 12 months (Colles, 1979; Colles, 1982; Turner, 1989). The effectiveness of the treatment is difficult to determine due to the fact that the horses had corrective shoeing during the treatment. Warfarin’s beneficial effects may have been due to its ability to alter erythrocyte morphology and reduce blood viscosity, possibly improving circulation through the navicular bone. Warfarin therapy is not used anymore because of difficulties stabilizing the dose and the potential risk of fatal hemorrhage.

Due to the difficulties associated with warfarin therapy, isoxsuprine hydrochloride was evaluated as a treatment for horses with navicular disease (Rose et al.,

1983; Turner and Tucker, 1989; Wilson and Bolhuis, 1996). Reported success rates for the treatment of horses with navicular disease in these studies ranged from 40% to 87%, with the best results occurring in horses affected less than one year. The horses were treated simultaneously with corrective shoeing. Isoxsuprine is a beta-adrenergic drug with both peripheral vasodilatory and rheologic properties. The beneficial response noted with isoxsuprine therapy is likely related to its ability to decrease blood viscosity and platelet aggregation, possibly improving navicular bone circulation. Harkins et al. (1998) reported absence of detectable pharmacological (peripheral vasodilatation) effects after oral administration of isoxsuprine in horses.

Although not specifically evaluated in the treatment of navicular disease, the administration of a corticosteroid into the navicular bursa is sometimes used to provide analgesia and mediate inflammation during the disease’s early stages. If utilized, corticosteroid therapy should be combined with other therapies (rest, corrective shoeing, etc.) to increase the likelihood of long-term success. It is not known if injection of corticosteroids into the distal interphalangeal joint (a site that is easier to inject than the navicular bursa) results in effective concentrations in the navicular bursa, however, clinical impressions suggest that many horses with navicular disease respond to this method of treatment (Crisman and Pleasant, 1997).

Hyaluronic acid and polysulphated glycosaminoglycan are now commonly used in the treatment for navicular disease. The chondroprotective and anti-inflammatory properties of these products would appear to be beneficial for the treatment of degenerative processes in the navicular bursa. Hyaluronic acid can be administered intrasynovially or intravenously. The use of hyaluronic acid in combination with a low dose of corticosteroid intrasynovially (navicular bursa or distal interphalangeal joint) has recently become popular, and may provide enhanced clinical benefits over the use of either product individually (Crisman and Pleasant, 1997). Results of a recent clinical study in humans demonstrated therapeutic synergy after intrasynovial use of this combination (Leardini et al., 1991), and a similar effect in horses is likely.

Polysulphated glycosaminoglycan can be administered intrasynovially or intramuscularly. One study evaluated polysulphated glycosaminoglycan (Adequan®) at a dose of 500 mg intramuscularly every 4 days for 7 treatments in horses with clinical signs of navicular disease of less than 12 months duration (Crisman et al., 1993). The treatment proved beneficial in treating some of the signs of lameness. Intrasynovial administration of PSGAG is not common due to the elevated risk of infection accompanying administration by this route.

Several oral supplement products containing chondroitin sulphate or glucosamine, or a combination of chondroitin sulphate and glucosamine, have

been marketed for use in horses in the past few years. Hanson et al. (1997a), reported significant improvement in horses with navicular disease when fed a compound containing chondroitin sulphate and glucosamine (Cosequin®). The use of these supplements appears to be a valid (and simple) method for aiding to the management of navicular disease.

Palmar digital neurectomy is sometimes used in the management of navicular disease. Palmar digital neurectomy is generally reserved for cases that have not responded to more conservative methods of treatment. Numerous methods of neurectomy have been described including the basic guillotine technique, a stripping technique, epineural capping, laser neurectomy, cryoneurectomy, silicone capping, intramedullary anchoring, and application of neurotoxic agents. All share the common goal of reducing painful neuroma formation and reinnervation. To date, no technique has been shown to be superior to the simple guillotine technique. Clinical results indicate that approximately 65% of neurectomized horses are sound for 1 year (Turner, 1989). It is important that correct trimming and shoeing be performed in conjunction with neurectomies. Correct trimming/shoeing should help slow the degenerative processes involving the navicular bone and navicular bursa and reduce complications such as navicular bone fracture and deep digital flexor tendon rupture.

Palmar digital neurectomy has been generally considered purely palliative in that it only eliminates pain perception from the palmar portion of the foot. Since the horse can no longer feel pain, concerns regarding the potential for overuse of already compromised tissues, and increased risks of catastrophic breakdowns have surfaced. However, a recent study reported that horses with navicular disease decreased the load on their navicular bones after palmar digital nerve analgesia (McGuigan and Wilson, 2001). This finding suggests that palmar digital neurectomy may reduce progressive damage to the navicular region in horses with navicular disease. This finding/suggestion warrants further investigation.

AIMS OF STUDY

Although histological and histochemical studies have been reported on the cartilage from the flexor surface of navicular bones of horses with navicular disease, to our knowledge there are no reports concerning the biochemical composition of the cartilage on the flexor surface of the navicular bone in normal or diseased states.

The purpose of this study was to determine the biochemical composition of normal equine navicular bone flexor surface cartilage.

The objectives of this study were as follows:

1) To determine the water content, total DNA content, total collagen content, total glycosaminoglycan content, and total chondroitin sulphate and keratan sulphate content of the cartilage of the flexor surface of the normal equine navicular bone.

2) To determine the water content, total DNA content, total collagen content, total glycosaminoglycan content, and total chondroitin sulphate and keratan sulphate content of the cartilage from the normal equine distal radiocarpal bone articular surface.

3) Compare the biochemical characteristics of the cartilage from these sites.

MATERIALS AND METHODS

Cartilage samples from the navicular bone flexor surface and distal radiocarpal bone articular surface were obtained from the right forelimbs of 8 horses, aged between 2-5 years. All horses were euthanised for reasons other than lameness.

Within 4 hours of euthanasia, the navicular and radial carpal bones were excised. Each bone was visually inspected, and only those with macroscopically smooth cartilage surfaces and no signs of degeneration were used. Each bone was sectioned midsagittally. One half of each bone was used for collection of the cartilage specimens. The other half of the bone was stored in a sealed plastic bag and frozen at -20°C. Approximately 1 cm x 1 cm squares of cartilage were removed with a scalpel from the flexor surface of the navicular bones and from the distal articular surface of the radiocarpal bones (area opposing the radial facet of the third carpal bone). The specimens were obtained from the area adjacent to the cut surface of the bones and, included the full thickness of the cartilage (Figure 5).

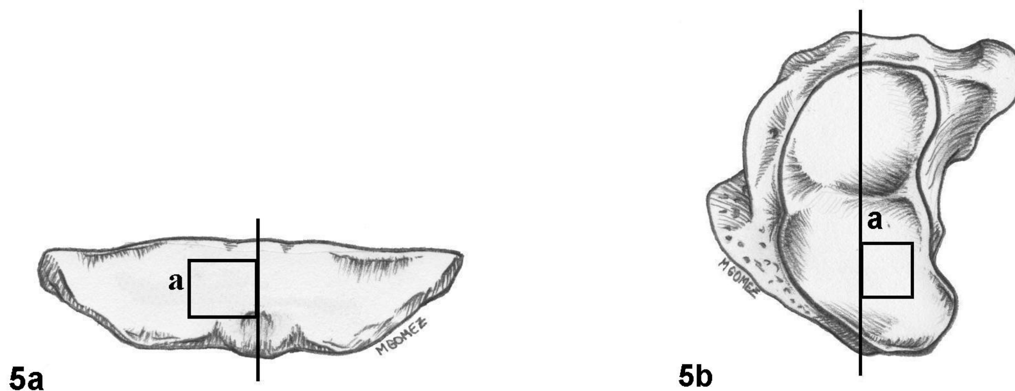

Figure 5: (5a) Diagram of the flexor surface of the navicular bone, and (5b) distal articular surface of the radiocarpal bone. Each bone was cut midsagittally, and cartilage specimens were sliced parallel to the cut surface (a).

Determination of water content:

Each cartilage specimen was immediately cut into approximately 2.5 mm by 2.5 mm squares. After recording the wet weight (ww), the samples were lyophilized for 24 hours and then reweighed to determine the dry weight (dw). Water content of the cartilage samples was calculated by [(ww-dw)/ww]*100% (Brama et al., 2000a). The lyophilized cartilage specimens were stored at -20°C until further analysis.

Determination of DNA content:

Cartilage cellularity was estimated by measuring the DNA content (Young-Jo et al., 1988; Richardson and Clark, 1991). The cartilage samples were incubated at 65°C for 4 hours in digest buffer (10mg ww cartilage/1 ml solution), which

contained papain[a] (0.5 mg/ml), 50 mM $NaPO_4$, 2mM N-acetylcysteine and 4mM EDTA disodium salt (pH 6.5). Aliquots of papain digested cartilage (100µl) were stained in 0.1 µg/ml of dye [b] solution. The fluorescence was measured using an excitation wavelength of 365 nm, and detection wavelength of 458 nm. The DNA concentrations were determined relative to a calf thymus DNA[a] standard curve (0 to 100 µg/ml) (appendix 2). Analyses were conducted in triplicate, and the mean value was used in subsequent calculations. Results were expressed as nanograms of DNA per milligram of dry weight of cartilage.

Determination of total collagen:

Total collagen content was determined on the basis of the 4-hydroxyproline content of the cartilage using a modified hydroxyproline assay (Reddy and Enwemeka, 1996). The cartilage samples were incubated in digest buffer with papain, as described previously. Aliquots of standard hydroxyproline[a] (0-30 µl of a stock solution of hydroxyproline 1 mg per ml), and papain-digested cartilage samples (250 µl) were oven dried at 37°C. Both standards and samples were mixed with sodium hydroxide (50 µl) and autoclaved at 120°C for 20 minutes. Buffered chloromide-T reagent (450 µl) was added to both standard and samples and oxidation was allowed to proceed for 25 minutes at room temperature. Ehrlich's aldehyde reagent[a] (500 µl) was then added, and the samples were incubated for 30 minutes at 65°C. Absorbance was measured at 550 nm in a spectrophotometer[d.]. The absorbance data was plotted against the

hydroxyproline standard curve (appendix 2). The analysis was conducted in triplicate, and the mean value was used for calculations. The collagen values were calculated assuming that 12.77% of the collagen is hydroxyproline (7.83 factor) (Vachon et al., 1990). The values were expressed as milligrams of collagen per milligrams of dry weight cartilage.

Determination of sulphated glycosaminoglycans:

A modified form of dimethylmethylene blue (DMMB) dye assay in conjunction with specific polysaccharidases digestion was used to determine the total glycosaminoglycans content and type (chondroitin sulphate and keratan sulphate) (Farndale et al., 1986). The cartilage samples were digested in the papain buffer described previously for 1 hour at 60° C. The digestion process was terminated by adding iodoacetic acid to a final concentration of 10mM.

Selective digestion of glycosaminoglycans with chondroitinase AC[a] and keratenase[a] followed the papain digestion. Digested cartilage samples were divided in 2 sub samples. Chondroitinase AC was added to the first sub sample to a final concentration of 0.05 units/ml. Water was added to the second sub sample to the same final volume. The samples were incubated at 37°C for 60 minutes. An aliquot (50 µl) was taken from each sub sample, and 1, 9-dymethylmethylene blue[c] (2.5 ml) was added. Absorbences were immediately measured with a spectrophotometer[d] at 525 nm. The analysis was conducted in

triplicate, and the mean value was used for calculations. The absorbence data was plotted against a shark chondroitin 4-sulphate standard curve (0-50μg/ml) (appendix 2).

The chondroitin content was calculated by subtracting the results obtained for the samples digested with chondroitinase AC from the samples treated with water. Chondroitin sulphate values were expressed as milligram per milligram of dry weight cartilage (Farndale et al., 1986; Palmer et al., 1995).

Keratenase or the same volume of water was added to the incubated sub samples to a final concentration of 0.1unit/ml and further digestion was performed at 37°C for 60 minutes. The reduction in sulphated glycosaminoglycans content assessed by dimethylmethylene blue spectophotometry due to keratenase digestion was plotted against a keratan sulphate standard curve (0-50μg/ml) (appendix 2). Each sample was analyzed in triplicate, and the mean value was used for calculations. The data was corrected for dilution and the content of glycosaminoglycans. Keratan sulphate values expressed as milligram per milligram of dry weight cartilage (Farndale et al., 1986; Palmer et al., 1995).

Statistical analysis:

A paired t test was used to compare water content, DNA content, total collagen content, total sulphated glycosaminoglycans, content of chondroitin sulphate, and keratan sulphate of the navicular bone flexor surface cartilage to the distal radiocarpal bone articular surface cartilage. A 0.05 level of significance was used in all statistical analysis.

[a] Sigma Chemical Co, St Louis, Mo.
[b] Hoechst 33258, Molecular Probes, Eugene, Ore.
[c] Aldrich Chemical Co, Milwaukee, Wis.
[d] Beckmann Industries Inc, Irving, Ca.

RESULTS

Eight horses with ages ranging from 23 to 60 months old (mean, 41.63 months) were utilized.

The mean concentrations, standard errors and mean differences for the different parameters based on wet weight and dry weight are presented in Table 1 and 2. The results of DNA content, total collagen, total glycosaminoglycans, chondroitin sulphate and keratan sulphate are presented based on dry weight to minimize the variation due to the water content.

Water content:

The mean water content was 68.32± 3.46 % for navicular bone flexor surface cartilage and 60.60± 4.09 % for distal radiocarpal bone articular surface cartilage. Significant difference was observed between the two types of cartilage (P= 0.002).

DNA content:

The mean for DNA content was 524.51± 92.89 ng/mg dry weight for navicular bone flexor surface cartilage and 508.80± 70.16 ng/mg dry weight for distal radiocarpal bone articular surface cartilage. There was not significant difference

(P=0.561) determined for DNA content between navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage.

Total collagen content:

The mean total collagen content was 0.1533± 0.0338 mg/mg dry weight for the navicular bone flexor surface cartilage and 0.1686± 0.0083 mg/mg dry weight for the distal radiocarpal bone articular surface cartilage. There was not significant difference (P= 0.287) observed for total collagen content between navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage.

Total glycosaminoglycans content:

The mean for total glycosaminoglycan content was 0.1018± 0.0197 mg/mg dry weight for the navicular bone flexor surface cartilage and 0.0919± 0.0191 mg/mg dry weight (9.19± 1.91 mg/100 mg dry weight) for the distal radiocarpal bone articular surface cartilage. There was not significant difference (P= 0.424) determined between navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage for total glycosaminoglycans content.

Chondroitin sulphate content:

The mean for chondroitin sulphate content was 0.0800± 0.0176 mg/mg dry weight in the navicular bone flexor surface cartilage and 0.0615± 0.0109 mg/mg dry weight for the distal radiocarpal bone articular surface cartilage. There was

not significant difference (P= 0.065) determined for chondroitin sulphate content between navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage.

Keratan sulphate content:

The mean for keratan sulphate content was 0.0092± 0.0037 mg/mg dry weight for the navicular bone flexor surface cartilage and 0.0074± 0.0018 mg/mg dry weight for the distal radiocarpal bone articular surface cartilage. There was not significant difference (P= 0.420) determined for keratan sulphate content between navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage.

Table 1: Measured parameters (mean ± s.d.) of the navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage based on wet weight.

Parameter	NB (mean± s.d.)	RC (mean± s.d.)	Mean difference
Water %	68.32± 3.46	60.60± 4.09	7.73*
DNA (ng/mg ww)	164.15± 21.35	198.29± 14.10	-34.14*
Collagen (mg/mg ww)	0.0481± 0.0099	0.0666± 0.0091	-0.0185*
GAG (mg/mg ww)	0.0320± 0.0055	0.0359± 0.0068	-0.0039
Chondroitin sulphate (mg/mg ww)	0.0250± 0.0046	0.0239± 0.0027	0.0011
Keratan sulphate (mg/mg ww)	0.0029± 0.0012	0.0028± 0.0009	0.0001

NB= navicular bone flexor surface cartilage; RC= distal radiocarpal bone articular surface cartilage; s.d.= standard deviation; ww=wet weight; GAG= glycosaminoglycans; *= significant differences ($P < 0.05$).

Table 2: Measured parameters (mean ± s.d.) of the navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage based on dry weight.

Parameter	**NB (mean± s.d.)**	**RC (mean± s.d.)**	**Mean difference**
Water %	68.32± 3.46	60.60± 4.09	7.73*
DNA (ng/mg dw)	524.51± 92.89	508.80± 70.16	15.71
Collagen (mg/mg dw)	0.1533± 0.0338	0.1686± 0.0083	-0.0153
GAG (mg/mg dw)	0.1018± 0.0197	0.0919± 0.0191	0.0099
Chondroitin sulphate (mg/mg dw)	0.0800± 0.0176	0.0615± 0.0109	0.0185
Keratan sulphate (mg/mg dw)	0.0092± 0.0037	0.0074± 0.0029	0.0018

NB= navicular bone flexor surface cartilage; RC= distal radiocarpal bone articular surface cartilage; s.d.= standard deviation; dw= dry weight; GAG= glycosaminoglycans; *= significant differences ($P < 0.05$).

DISCUSSION

The amount of water was significantly higher (P=0.002) in the navicular bone flexor surface cartilage (68.32± 3.46%) than in the distal radiocarpal bone articular surface cartilage (60.60± 4.09%). These percentages are both in agreement with the previously reported values of 58 to 75% for equine hyaline articular cartilage (Todhunter, 1996; Brama et al., 2000a). It has also been inferred from histological studies that the cartilage on the flexor surface of the navicular bone is fibrocartilage (Smith, 1893; Pryer, 1934; Wilkinson, 1953), and that such fibrocartilage usually has lower water content than hyaline cartilage (Dijkgraaf et al., 1995). Our results are in disagreement with this suggestion.

Significant differences in the water content of cartilage between two different sites within a joint, and between joints have been reported by Brama et al. (1999), suggesting that different loading characteristics can affect water contents. The collagen fibrils constitute a network that retains proteoglycans whose GAG chains create an osmotic pressure drawing water in to the cartilage. In the present study, the cartilage from the navicular bone flexor surface has a slightly higher GAG content compared to the cartilage of the distal radiocarpal bone articular surface. This may help explain the higher water content in the cartilage from the flexor surface of the navicular bone.

The content of DNA is used to estimate the number of chondrocytes in the cartilage, and thereby the cellularity of the tissue. The values obtained for DNA in the present study, 524.51± 92.89 ng/mg dw for the cartilage of the flexor surface of the navicular bone, and 508.80± 70.16 ng/mg dw for the cartilage of the distal radiocarpal bone articular surface are similar to previously reported values for equine hyaline articular cartilage. Brama et al. (2000b) sampled cartilage from 2 different sites from the proximal articular surface of the first phalanx in neonatal foals and obtained values of 1173 and 1282 ng /mg dw. The DNA content dropped to 668 and 551 ng /mg dw in yearling horses. Platt et al. (1998) also reported a similar decrease of cartilage cellularity with age, suggesting a reduction in the rate of cell proliferation. In addition, Brama et al. (2000a) reported DNA contents of 363± 8.7 ng/mg dw for the proximal articular surface of first phalanx, of 425±11.2 ng/mg dw for the distal metacarpus, and of 358±11.7 ng/mg dw for the proximal sesamoid bone articular surface in horses aged 5 to 9 years old. Finally, Dijkgraaf et al. (1995) suggested, based on De Bond (1985), that the cellularity of fibrocartilage (0.01% to 0.1%) is less than for hyaline cartilage (1 to 12%). Our findings determined no significant differences between the total DNA content of the navicular bone flexor surface cartilage and the distal radiocarpal bone articular surface.

The amount of total collagen obtained in the present study was 15.33± 3.38mg/ 100 mg dw for the cartilage of the flexor surface of the navicular bone, and

16.86± 0.83 mg/ 100 mg dw for the distal radiocarpal bone articular surface cartilage. The total collagen values obtained here are lower than the values of 48 to 63 mg/100 mg dw previously reported for equine hyaline articular cartilage (Vachon et al., 1990; Vachon et al., 1991; Barr et al., 1994; Brama et al., 1999; Brama et al., 2000a; Brama et al., 2001). This may be partially explained by differences in the preservation and analysis methods being used. For instance, Vachon et al. (1991) reported a collagen content of 55.6 mg/100 mg dw in distal radiocarpal bone articular cartilage, but used a slightly different colorimetric method (Kivirikko et al., 1967) to determine hydroxyproline than the one used here (Reddy and Enwemeka, 1996). There are no previous reports of the usage of the Reddy and Enwemeka (1996) method for the determination of hydroxyproline for total collagen content in horses, and it is possible that the technique underestimated hydroxyproline. These differences may also be explained by the different desiccation method used. Samples subjected to 96 hours of vacuum desiccation yielded hydroxyproline content 8.5% less than similar oven-dried (110ºC for 24 hours) samples (Johnson et al., 1980). The authors suggested that the vacuum drying did not yield a true dry weight and that 8.5% of the “dry” weight was still water. In the present study, the samples were dried by lyophilization for 24 hours. Vachon et al. (1990) instead of oven driying samples at 110ºC for 18 hours. It could be also possible that the papain digestion process was not able to completely digest the cartilage samples before the determination of total collagen.

Nevertheless, although the total collagen contents reported here likely underestimate the amount of collagen present in both cartilages, the data can still be used for comparison. No statistical differences were determined between the cartilage from the flexor surface of the navicular bone and the distal radiocarpal articular surface cartilage. Fibrocartilage contains about 60% total collagen based on dry weight with predominantly type I collagen (Dijkgraaf et al., 1995,) and hyaline cartilage is composed of approximately 50% of collagen based on dry weight with 85 to 90% of it being type II collagen (Todhunter, 1996).

To further characterize the cartilage of the flexor surface of the navicular bone, the type and proportions of the collagen present should also be determined. We performed a pilot study in four horses with a different range of age than the ones used in the present study. Results suggested that the collagen present in cartilage of the flexor surface of the navicular bone was basically 75% type I with less than 25% being type II collagen.

The values obtained for total glycosaminoglycans were 10.18±1.97 mg/100mg dw for navicular bone flexor surface cartilage, and 9.19±1.91 mg/100mg dw for the distal radiocarpal articular surface cartilage. The total GAG content for the navicular bone flexor surface cartilage and for distal radiocarpal bone articular surface cartilage were similar to previously reported values for distal radiocarpal

bone articular surface cartilage (7.32 mg/100 mg dw) (Vachon et al., 1990; Vachon et al., 1991), and fetlock joint (4.24 mg/100 mg to 14.1 mg/100 mg dw) (Platt et al., 1998; Brama et al., 2000a; Brama et al., 2000b). It has been suggested that fibrocartilage contains less GAG than hyaline cartilage (Dijkgraaf et al., 1995).

Chondroitin sulphate is the most abundant glycosaminoglycan in cartilage. The amount of chondroitin sulphate determined for the cartilage was 8.0± 1.76 mg/100mg dw for the flexor surface of the navicular bone and 6.15±1.09 mg/100mg dw for the distal radiocarpal bone articular surface cartilage. These results are similar to the previously reported content 5.33 mg/100mg dw for cartilage of the distal radiocarpal bone articular surface (Vachon et al.,1990). No differences were determined between the chondroitin sulphate content of the navicular bone flexor surface cartilage and the distal radiocarpal bone articular surface cartilage. Differences have been reported in the proportion of chondroitin-4 sulphate and chondroitin-6 sulphate in fibrocartilage and articular cartilage. The present study determined total chondroitin sulphate content without differentiating types.

Keratan sulphate content was not significantly different in the navicular bone flexor surface cartilage (0.92± 0.37 mg/100 mg dw) than in the distal radiocarpal articular surface cartilage (0.74± 0.18 mg/100mg dw). The keratan sulphate

values obtained from both sites were less than the keratan sulphate content reported for the distal radiocarpal articular surface cartilage (1.99 mg/100mg dw) in horses (Vachon et al., 1990; Vachon et al., 1991). These authors determined keratan sulphate on the basis of the content of glucosamine in the cartilage. The glycoproteins and hyaluronic acid present in cartilage also contain small amounts of glucosamine, resulting in an overestimation of keratan sulphate contents. The keratan sulphate content was reported to be smaller in fibrocartilage than in articular cartilage (Dijkgraaf et al., 1995).

In general, fibrocartilage has been described has having less water content, higher total collagen content, less total GAG content and less keratan sulphate than articular cartilage. Except for the water content, and DNA content and collagen content based on wet weight, no significant differences were found between the biochemical constituents of navicular bone flexor surface cartilage and the distal radiocarpal bone articular surface cartilage. These results allow us to conclude that the cartilage from flexor surface of the navicular bone has biochemical characteristics similar to the distal radiocarpal articular surface cartilage. If the cartilage of the flexor surface of the navicular bone is indeed fibrocartilage based on the collagen type I, as suggested by our pilot study, it would differ biochemically from the classic fibrocartilage. Further studies are neededddde4 to determine the type and proportions of collagen present in the flexor surface of the navicular bone.

These findings establish a comparison basis to assess the flexor surface cartilage of the navicular bone in aging, disease, and repair.

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APPENDIX 1

Identification and Signalment

Horse	Number	Case Number	Age (months)	Breed	Sex
Meridens Angus	1	62074	46	Morgan	G
Kyhrss	2	51721	58	Arab	G
Howie	3	63228	60	Quarter Horse	M
Huey	4	62385	23	Quarter Horse	M
Keena	5	64079	42	Mixed	G
Peppy	6	65052	52	Quarter Horse	G
Try to Beat Me Doc	7	64921	23	Quarter Horse	G
Milo	8	65404	29	Appaloosa	G

G= Gelding; M= Mare.

APPENDIX 2

Standard Curves

2.1 DNA Standard Curve:

DNA ng/ml	Average Emission
0	0.00
50	0.40
100	0.90
200	1.45
300	2.20
400	2.75
500	3.40
1000	8.15
5000	40.50

$y=0.0081x-0.1816$

$R^2=0.9996$

2.2 Hydroxyproline Standard Curve:

Hydroxyproline μg	**Average Emission**
0	0.00085
2.5	0.05545
5	0.23785
7.5	0.28960
10	0.41445
15	0.42305
20	0.53200
25	0.58815
30	0.73035
40	0.81340
50	1.08330
60	1.11585

$y=0.0182x+0.1221$

$R^2=0.9574$

2.3 Chondroitin 4 Sulphate Standard Curve:

Chondroitin Sulphate µg/ml	**Average Emission**
0	0.00190
25	0.02450
50	0.05670
75	0.09405
100	0.12935
150	0.19575
200	0.25705
300	0.34600
400	0.40915
500	0.44605

$y=0.0009x+0.0269$

$R^2=0.9651$

2.4 Keratan Sulphate Standard Curve:

Keratan Sulphate μg/ml	**Average Emission**
0	0.00040
2.5	0.04055
5	0.07040
7.5	0.10075
10	0.13615
15	0.20370
20	0.24985
30	0.34500

$y=0.0115x+0.0136$

$R^2=0.9918$

APPENDIX 3

Averages values for water content, DNA, total collagen, total glycosaminoglycans, chondroitin sulphate and keratan sulphate in navicular bone flexor surface cartilage and distal radiocarpal bone articular surface cartilage

3.1 Water Content:

Horse	Source	% water
Meridens Angus	0	67.0143
Kyhrss	0	69.4937
Howie	0	68.4018
Huey	0	68.7357
Keena	0	73.7119
Peppy	0	61.8698
Try to Beat Me Doc	0	66.4797
Milo	0	70.8591
Meridens Angus	1	55.9796
Kyhrss	1	56.9740
Howie	1	67.5419
Huey	1	58.2790

Keena	1	61.8158
Peppy	1	59.3205
Try to Beat Me Doc	1	59.3095
Milo	1	65.5420

0= navicular bone flexor surface cartilage; 1= distal radiocarpal bone articular surface cartilage.

3.2 DNA Content:

Horse	Source	DNA (ng/mg ww)	DNA (ng/mg dw)
Meridens Angus	0	119.8333	363.2890
Kyhrss	0	166.0667	544.3681
Howie	0	194.6333	615.9637
Huey	0	166.7000	533.1958
Keena	0	167.9333	638.8200
Peppy	0	158.6333	416.0305
Try to Beat Me Doc	0	179.2667	534.8000
Milo	0	160.1667	549.6290
Meridens Angus	1	198.0667	449.9425
Kyhrss	1	212.3333	493.5000
Howie	1	196.2000	604.4714
Huey	1	170.1000	407.7082
Keena	1	217.3000	569.0837
Peppy	1	194.6333	478.4560
Try to Beat Me Doc	1	195.6000	480.7014
Milo	1	202.1000	586.5120

0= navicular bone flexor cartilage; 1= distal radiocarpal bone articular surface cartilage; ww= wet weight; dw= dry weight.

3.3 Total Collagen Content:

Horse	Source	Collagen mg/mg ww	Collagen mg/mg dw
Meridens Angus	0	0.040532440	0.122878896
Kyhrss	0	0.038547692	0.126359693
Howie	0	0.043188330	0.136679801
Huey	0	0.042287736	0.135258818
Keena	0	0.049934176	0.189850095
Peppy	0	0.048064154	0.126052653
Try to Beat Me Doc	0	0.069230967	0.206534203
Milo	0	0.053140747	0.182358136
Meridens Angus	1	0.073888813	0.167851146
Kyhrss	1	0.076642220	0.178130014
Howie	1	0.051505912	0.158684261
Huey	1	0.074967231	0.179686956
Keena	1	0.059427692	0.155634288
Peppy	1	0.069012989	0.169650617
Try to Beat Me Doc	1	0.069139187	0.169914646
Milo	1	0.058228813	0.168985135

0= navicular bone flexor cartilage; 1= distal radiocarpal bone articular surface cartilage; ww= wet weight; dw= dry weight.

3.4 Total Glycosaminoglycans Content:

Horse	**Source**	**GAG mg/mg ww**	**GAG mg/mg dw**
Meridens Angus	0	0.033510638	0.101591473
Kyhrss	0	0.023992908	0.078648974
Howie	0	0.026968085	0.085346957
Huey	0	0.035762411	0.114387336
Keena	0	0.035	0.133140343
Peppy	0	0.031882979	0.083616037
Try to Beat Me Doc	0	0.040946809	0.122155111
Milo	0	0.027900709	0.095744256
Meridens Angus	1	0.040202128	0.091326047
Kyhrss	1	0.041273050	0.095925835
Howie	1	0.034324468	0.105750052
Huey	1	0.023315603	0.055884547
Keena	1	0.039675532	0.103905653
Peppy	1	0.042632979	0.104802172
Try to Beat Me Doc	1	0.028492908	0.07002342
Milo	1	0.037202128	0.107963845

0= navicular bone flexor cartilage; 1= distal radiocarpal bone articular surface cartilage; GAG= glycosaminoglycans; ww= wet weight; dw= dry weight

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3.5 Chondroitin Sulphate Content:

Horse	**Source**	**Chondroitin sulphate mg/mg ww**	**Chondroitin sulphate mg/mg dw**
Meridens Angus	0	0.026443262	0.080165885
Kyhrss	0	0.020446809	0.067024828
Howie	0	0.018687943	0.059142467
Huey	0	0.030294326	0.096897473
Keena	0	0.026833333	0.102074263
Peppy	0	0.021283688	0.055818424
Try to Beat Me Doc	0	0.031365248	0.093570794
Milo	0	0.024886525	0.085400761
Meridens Angus	1	0.023496454	0.053376236
Kyhrss	1	0.024900709	0.057873633
Howie	1	0.022941489	0.0706803
Huey	1	0.019446809	0.046611537
Keena	1	0.022452128	0.058799535
Peppy	1	0.026817376	0.065923596
Try to Beat Me Doc	1	0.023446809	0.057622259
Milo	1	0.02806383	0.081443701

0= navicular bone flexor cartilage; 1= distal radiocarpal bone articular surface cartilage; GAG= glycosaminoglycans; ww= wet weight; dw= dry weight

3.6 Keratan Sulphate Content:

Horse	Source	Keratan sulphate mg/mg ww	Keratan sulphate mg/mg dw
Meridens Angus	0	0.004249275	0.012882182
Kyhrss	0	0.003078261	0.010090568
Howie	0	0.000408696	0.001293415
Huey	0	0.002991304	0.009567793
Keena	0	0.002455072	0.00933912
Peppy	0	0.002617391	0.006864349
Try to Beat Me Doc	0	0.004188406	0.012495117
Milo	0	0.003150725	0.010812047
Meridens Angus	1	0.001631884	0.003707105
Kyhrss	1	0.00286087	0.006649165
Howie	1	0.004195652	0.01292636
Huey	1	0.00316087	0.007576204
Keena	1	0.002098551	0.005495863
Peppy	1	0.002353623	0.005785775
Try to Beat Me Doc	1	0.003982609	0.009787554
Milo	1	0.002382609	0.00691454

0= navicular bone flexor cartilage; 1= distal radiocarpal bone articular surface cartilage; GAG= glycosaminoglycans; ww= wet weight; dw= dry weight

VITA

Lucia Carolina E. Vits was born on August 16, 1968 in Concepción, Chile. She attended elementary, junior high, and high school at Alliance Française in Concepción. She enrolled in the Veterinary Sciences program of the Universidad Austral de Chile (Valdivia, Chile), and obtained a degree in Veterinary Medicine in 1993. Following two years of field service in the dairy area, she was appointed as faculty member of the surgery department of the Veterinary Hospital of the Universidad Austral. She was then appointed as Large Animal Surgery Resident at the Virginia-Maryland College of Veterinary Medicine (Blacksburg, VA) in July 1999, and completed her residency program in October 2002.