

Assessment of Glycosaminoglycan Concentration in Equine Synovial Fluid as a Marker of Joint Disease

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ABSTRACT

A modification of a colorimetric assay was used to determine synovial fluid total and individual sulphated-glycosaminoglycan concentration in various clinical presentations of joint disease in horses. Concentrations of synovial fluid and serum sulphated-glycosaminoglycan (GAG) were measured by the 1,9-dimethylmethylene blue (DMMB) dye assay in normal horses ($n = 49$), horses with acute ($n = 26$) or chronic ($n = 27$) joint disease (defined by clinical, radiographic, and clinicopathological parameters), and horses with cartilaginous lesions at diagnostic arthroscopy, but with normal radiographs and synovial fluid ($n = 9$). Horses with acute joint disease were subdivided into moderate acute ($n = 21$) and severe acute ($n = 5$) joint disease on the basis of synovial fluid analysis and clinical examination. Horses with chronic joint disease were subdivided into mild chronic ($n = 9$), moderate chronic ($n = 10$), and severe chronic ($n = 8$) joint disease on the basis of synovial fluid analysis, clinical examination, and radiographic findings. The concentrations of chondroitin sulphate (CS) and keratan sulphate (KS) were analyzed in each sample following sequential enzymatic digestion of the sample with chondroitinase or keratanase. In addition, the concentration of hyaluronate (HA) in each sample was determined by a colorimetric assay following digestion of the sample with microbial hyaluronidase. Synovial fluid sulphated-GAG concentration (mean \pm SEM) was significantly increased ($P < 0.05$) in horses with moderate acute ($106.4 \mu\text{g/mL} \pm 11.7 \mu\text{g/mL}$), severe

acute ($159.0 \mu\text{g/mL} \pm 47.7 \mu\text{g/mL}$), moderate chronic ($78.3 \mu\text{g/mL} \pm 9.4 \mu\text{g/mL}$), and severe chronic ($122.9 \mu\text{g/mL} \pm 22.5 \mu\text{g/mL}$) joint pathology and in horses with detectable cartilaginous arthroscopic lesions ($67.2 \mu\text{g/mL} \pm 11.6 \mu\text{g/mL}$), but without synovitis or radiographic abnormalities as compared with synovial fluid sulphated-GAG of normal horses ($25.6 \mu\text{g/mL} \pm 2.6 \mu\text{g/mL}$). Synovial fluid CS concentrations (mean \pm SEM) were significantly increased in horses with moderate acute ($74.9 \mu\text{g/mL} \pm 7.7 \mu\text{g/mL}$), severe acute ($94.8 \mu\text{g/mL} \pm 19.8 \mu\text{g/mL}$), moderate chronic ($45.5 \mu\text{g/mL} \pm 7.8 \mu\text{g/mL}$), and severe chronic ($87.9 \mu\text{g/mL} \pm 17.7 \mu\text{g/mL}$) joint pathology and in horses with detectable cartilaginous arthroscopic lesions ($47.9 \mu\text{g/mL} \pm 7.5 \mu\text{g/mL}$), but without synovitis or radiographic abnormalities as compared with normal horse synovial fluid CS concentration ($12.1 \mu\text{g/mL} \pm 2.7 \mu\text{g/mL}$). In contrast, the synovial fluid KS concentration (mean \pm SEM) was increased only in horses with moderate chronic ($25.2 \mu\text{g/mL} \pm 5.1 \mu\text{g/mL}$) and severe chronic ($30.3 \mu\text{g/mL} \pm 5.7 \mu\text{g/mL}$) joint disease when compared with synovial fluid KS concentration in normal horses ($7.6 \mu\text{g/mL} \pm 1.2 \mu\text{g/mL}$). When compared with normal, there was no significant difference in serum levels of sulphated-GAGs in any category of joint disease and no significant difference in the mean concentration of synovial fluid HA in any category of joint disease. As measured by standard synovial fluid analysis, there were significant correlations ($P < 0.0001$) between the concentration of synovial fluid sulphated-GAG and synovial fluid

white blood cell count ($r = 0.38$) and total protein ($r = 0.54$). There was no correlation between the concentrations of synovial fluid sulphated-GAG and synovial fluid HA or between the concentrations of synovial fluid sulphated-GAG and serum sulphated-GAG.

RÉSUMÉ

Les concentrations totale et partielle de glycosaminoglycan sulfaté dans le liquide synovial de chevaux atteints de différentes maladies articulaires ont été déterminées à l'aide d'une épreuve de colorimétrie modifiée. Les concentrations sériques et synoviales de glycosaminoglycan sulfaté (GAG) ont été mesurées par l'épreuve de colorimétrie au bleu de 1,9-diméthylméthylène chez des chevaux normaux ($n = 49$), des chevaux avec des problèmes articulaires aigus ($n = 26$) ou chroniques ($n = 27$) (tels que définis par des critères cliniques, radiographiques et clinicopathologiques), et chez des chevaux avec des lésions cartilagineuses détectées lors d'une arthroscopie, mais ne présentant aucune altération radiographique ou d'altération du liquide synovial ($n = 9$). À la suite d'un examen clinique et de l'analyse du liquide synovial, les chevaux avec des problèmes articulaires aigus ont été subdivisés en deux groupes : aigu modéré ($n = 21$) et aigu sévère ($n = 5$). Après un examen clinique et radiologique ainsi qu'une analyse du liquide synovial, le groupe avec des problèmes chroniques a été subdivisé en groupe chronique léger ($n = 9$), chronique modéré ($n = 10$) et chronique sévère ($n = 8$). Les

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concentrations de sulfate de chondroïtine (CS) et de sulfate de kératine (KS) ont été mesurées dans chaque échantillon à la suite d'une digestion enzymatique séquentielle de l'échantillon avec de la chondroïtinase ou de la kératanase. De plus, la concentration d'hyaluronate (HA) de chaque échantillon a été déterminée par un essai de colorimétrie utilisant la digestion de l'échantillon avec de la hyaluronidase microbienne. Comparativement aux chevaux normaux ($25,6 \pm 2,6 \mu\text{g/mL}$), la concentration de GAG sulfaté dans le liquide synovial était augmentée de façon significative ($P < 0,05$) chez les chevaux des groupes aigu modéré ($106,4 \pm 11,7 \mu\text{g/mL}$), aigu sévère ($159,0 \pm 47,7 \mu\text{g/mL}$), chronique modéré ($78,3 \pm 9,4 \mu\text{g/mL}$), chronique sévère ($122,9 \pm 22,5 \mu\text{g/mL}$) et chez les chevaux avec des lésions détectables par arthroscopie mais sans synovite ou anomalie radiographique ($67,2 \pm 11,6 \mu\text{g/mL}$). Les concentrations de CS dans le liquide synovial étaient augmentées de façon significative, comparativement aux chevaux normaux ($12,1 \pm 2,7 \mu\text{g/mL}$), chez les chevaux avec atteinte articulaire de type aigu modéré ($74,9 \pm 7,7 \mu\text{g/mL}$), aigu sévère ($94,8 \pm 19,8 \mu\text{g/mL}$), chronique modéré ($45,5 \pm 7,8 \mu\text{g/mL}$), chronique sévère ($87,9 \pm 17,7 \mu\text{g/mL}$) et chez les chevaux avec des lésions détectables par arthroscopie mais sans synovite ou anomalie radiographique ($47,9 \pm 7,5 \mu\text{g/mL}$). Une augmentation du KS dans le liquide synovial n'a été notée que chez des chevaux avec atteinte chronique modérée ($25,2 \pm 5,1 \mu\text{g/mL}$) et chronique sévère ($30,3 \pm 5,7 \mu\text{g/mL}$) lorsque comparée aux chevaux normaux ($7,6 \pm 1,2 \mu\text{g/mL}$). Aucune différence dans les concentrations sériques de GAG sulfaté n'a été notée entre les différents groupes avec atteintes articulaires et les chevaux normaux; de même que dans les concentrations moyennes d'HA dans le liquide synovial des chevaux avec maladie articulaire. Tel que mesuré par analyse standard du liquide synovial, il y a une corrélation significative ($P < 0,0001$) entre la concentration de GAG sulfaté du liquide synovial et le comptage leucocytaire ($r = 0,38$) ainsi qu'avec les protéines totales ($r = 0,54$) du liquide synovial. Il n'y a

aucune corrélation entre les concentrations de GAG sulfaté et d'HA du liquide synovial ou entre les concentrations de GAG sulfaté sérique et du liquide synovial.

(Traduit par Docteur Serge Messier)

INTRODUCTION

Articular cartilage of diarthrodial joints consists of chondrocytes embedded within an abundant extracellular matrix of collagen, proteoglycans, noncollagenous proteins, and water. The matrix provides compressive stiffness, which enables the articular cartilage to withstand load. The compressive stiffness is a function of the polyanionic character of the matrix glycosaminoglycans (GAG), which are covalently linked to proteoglycan core protein. The sulphate and carboxyl groups of the GAG, chondroitin sulphate (CS), and keratan sulphate (KS), attract and trap water, resulting in an expansion of the cartilage matrix. Tightly packed Type II, IX, and XI collagen fibrils resist this expansion, thus providing the articular cartilage with the capacity to resist compressive forces. Alterations in proteoglycan and glycosaminoglycan structure, concentration, or composition due to inflammation or altered mechanics result in a change of compressive stiffness of the articular cartilage and contribute to cartilage damage.

Evaluations of synovial fluid analysis, cytology, radiography, and lameness examinations provide the current basis of categorization of joint disease. More sensitive markers are needed to detect radiographically silent cartilage lesions and to distinguish the gradations of moderate and severe acute synovitis, and early and late stages of degenerative joint disease. The 1,9-dimethylmethylene blue (DMMB) dye assay allows an inexpensive, simple, rapid, and direct measurement of sulphated-GAG present in synovial fluid using a small amount of sample. Additional incubation of samples with glycosidases directed against CS or KS (chondroitinase ABC lyase and keratanase, respectively) allow for the assessment of individual sulphated-GAGs and may provide some insight into the pathophysiology of the arthritis.

Increases in the concentration of synovial fluid sulphated-GAG have

been reported in people, horses, and rabbits with traumatic, or osteoarthritic synovitis using the DMMB spectrophotometric assay (1-4). Glycosaminoglycan concentration in synovial fluid increases with increasing Mankin scores of osteoarthritis in humans, suggesting that increases in the total amount of synovial fluid GAG reflect progressive destruction of osteoarthritic cartilage (3). Similar conditions may exist in equine osteoarthritis, and thus explain the large variations in synovial fluid GAG concentration reported when osteoarthritis was considered a single entity (1,2,5).

The glycosaminoglycans of importance in maintaining mature articular cartilage structure and function are chondroitin-6-sulphate (C6S), keratan sulphate (KS), and hyaluronate (HA). Chondroitin sulphate has been isolated in high concentrations from all layers of the normal articular cartilage matrix and in osteoarthritic cartilage, where it is thought to reflect an attempt at repair, often resulting in "supersulfation" of the CS moiety (6). Keratan sulphate matrix concentrations increase and become more heterogenous in size during the aging process and with increasing depth from the articular cartilage surface (7). The migration of KS from the cartilage matrix to the joint fluid is less diffusion-limited than that of the larger CS chains because it is relatively small. However, its location on the proteoglycan core protein shields it from enzymatic attack due to the steric hinderance of the large CS chains. Therefore, its presence in the synovial fluid may reflect more chronic processes (i.e., degradation after the release of CS), or alternatively reflect a stimulation of both CS and KS synthesis that cannot be retained within the damaged matrix (8,9).

Hyaluronate (HA) is a high molecular weight unsulphated-GAG normally present within the articular cartilage matrix and the synovial fluid compartment. The molecular weight of HA and its ability to form mucin precipitate were significantly reduced in the synovial fluid of horses with various arthritides in some studies, but were unchanged in others (5,10,11,12,13). A decrease in HA concentration within the synovial fluid may reflect the presence of hyaluronidases

Table 1. Assignment criteria for joint disease grouping

	Acute joint disease (n = 26)		Chronic joint disease (n = 27)			Cartilage lesion (n = 9)	Normal (n = 49)
	Moderate (n = 21)	Severe (n = 5)	Mild (n = 9)	Moderate (n = 10)	Severe (n = 8)		
Grade of lameness (≤ 5)	1-3	3-5	1-3	1-3	1-3	0	0
Joint effusion	Moderate	Marked	Mild	Moderate	Moderate	0	0
Radiographs	(\pm)Bony chip or defect	(\pm)Osteomyelitis	Dissecan or fragment	Narrow joint space (\pm)Osteophytes	Narrow joint space Osteophytes Subchondral Sclerosis	Normal	Normal
Total protein (g/L)	25-60	20-80	<30	20-40	>30	<25	<2.5
WBC count ($\times 10$ cells/L)	1.0-15.0	>15.0	0-1.5	0-2.0	0-6.0	<1.0	<1.0
Mucin precipitate	Fair	Poor	Fair	Fair	Poor	Good	Good

or oxygen-derived free radicals released from invading neutrophils during the respiratory burst, or a decrease in synoviocyte HA production while they are simultaneously upregulating their production of inflammatory mediators. Given its large size, cartilaginous HA likely has a protracted diffusion from the matrix in cartilage breakdown and may not contribute significantly to the synovial fluid HA concentration.

The method of measuring individual sulphated-GAGs using specific glycosidases against CS or KS has been previously reported, but has not been applied to individual samples (2,14). Other techniques for the measurements of individual GAGs include electrophoresis, ion-exchange chromatography and immunoassay (1,15,16). While these techniques may be more sensitive, they are time consuming and require more technical expertise. The DMMB assay offers advantages for rapid sample analysis that can potentially be performed on clinical samples.

The purpose of this present study was to determine the concentration of both total and individual sulphated-GAGs and of HA in the synovial fluid and serum of clinical samples from horses presented to the Ohio State University for orthopedic evaluation using a modification of the DMMB assay. We sought to determine the usefulness of the DMMB assay to characterize the degree of joint disease.

MATERIALS AND METHODS

GROUP SELECTION AND SAMPLE COLLECTION

Synovial fluid samples from a total of 62 horses were collected by an appropriate standard aseptic technique

from various joints (65.3% antebrachio-carpal or middle carpal, 13.9% tarsocrural, 9.7% metacarpophalangeal or metatarsophalangeal, 8.3% femoropatellar or femorotibial, and 2.8% distal interphalangeal) of horses presented to the Ohio State University for orthopedic examination. The clinical categories of joint abnormalities were made on the basis of clinical and lameness examinations, radiographic interpretation, and routine clinicopathological analysis, which included synovial fluid white blood cell (WBC) total and differential counts, total protein, color/clarity, specific gravity, and mucin precipitate test (17). The specific criteria for each category were well defined and are listed in Table I. Horses were assessed at the time of presentation for lameness graded on a standard scale of 0 to 5 at the trot, with grade 0 as sound and grade 5 as nonweight bearing with joint effusion. Radiographs were independently evaluated by the attending radiologist without knowledge of the study criteria. Synovial fluid analysis was also performed independently by the Ohio State University Veterinary Hospital Clinicopathological Laboratory without knowledge of the study criteria.

Categories of joint disease included horses with acute (n = 26) and chronic (n = 27) joint disease. Horses with acute joint disease were subdivided into moderate (n = 21) and severe (n = 5) joint disease. Horses with chronic joint disease were subdivided into mild (n = 9), moderate (n = 10), and severe (n = 8) categories (Table I) (17). Synovial fluid was also collected from horses (n = 49) donated to the Ohio State University for nonmusculoskeletal problems. Samples from these horses were only considered for inclusion into the

normal category after meeting the criteria included in Table I and after gross examination of the synovial membrane and articular cartilage following euthanasia. In addition, synovial fluid was collected from horses (n = 9) with cartilage lesions detected during diagnostic arthroscopy, but without clinical synovitis or radiographic lesions. Diagnostic arthroscopy was performed on these horses for a research protocol or by request of the referring veterinarian or the client. Approximately 2.0 mL of synovial fluid was collected into sterile polypropylene vials and centrifuged at $10\,000 \times g$ for 20 min to remove cellular debris. Following centrifugation, supernatant fluids were transferred to new sterile vials and stored at -80°C until analysis. In addition, approximately 20 mL of blood from each horse was collected at the time of arthrocentesis for determination of serum sulphated-GAGs. Blood samples were collected into sterile serum tubes, then centrifuged and the serum aliquoted into sterile vials. Serum samples were stored at -80°C until analysis.

DMMB-GAG COMPLEX INTERACTION

The 1,9 dimethylmethylene blue (DMMB) dye assay for assessment of total and individual sulphated-GAGs (CS and KS) was adapted and modified (see below) from a method previously described (2,14). The absorbance of the DMMB-GAG complexes was determined at 525 nm in a spectrophotometer (Beckmann Industries, Inc., Model 35, Irving, California).

DMMB-GAG COLOR STABILITY

DMMB-GAG complexes have a tendency to aggregate and precipitate over time. To determine the stability of

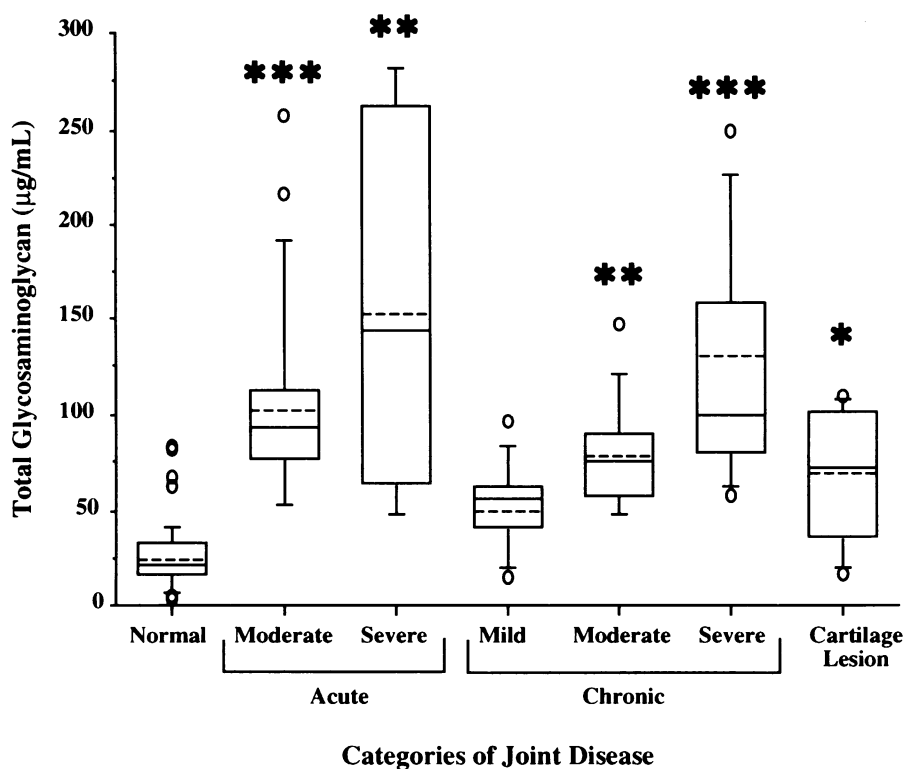


Fig. 1. Box plot of synovial fluid total sulphated-glycosaminoglycan (GAG) concentration measured by DMMB assay and grouped according to joint disease status (Table I). Mean of values represented by dashed bar. Median of values represented by solid bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

the DMMB-GAG complex in this laboratory, 100 μL of standard solutions of CS and KS at 5, 10, and 20 $\mu\text{g}/\text{mL}$ were added to 2.5 mL of DMMB reagent. The absorbance changes were measured over a 30-min time period.

STANDARD CURVES

The absorbance of the DMMB-complexes with C6S, KS, and human umbilical cord HA (Sigma Chemical Co., St. Louis, Missouri) were plotted against known concentrations ranging from 0.625 to 50.0 $\mu\text{g}/\text{mL}$. The linear portion of the C6S curve was used to determine the concentration of both total and individual sulfated-GAG in all samples.

SAMPLE GLYCOSAMINOGLYCAN DETERMINATION

All synovial fluid samples were initially diluted 1:5 (2). However, high concentrations of sulphated-GAG in nonnormal samples resulted in precipitation of the sample GAG with the DMMB reagent and falsely lowered the actual value of sulphated-GAG. Therefore, samples were subsequently diluted according to clinical category

(1:5 normal, 1:15 acute and severe, 1:10 chronic and cartilaginous lesions) with phosphate buffered saline (PBS, pH 7.0). Corresponding serum samples from each animal were diluted 1:10 in the same PBS buffer. The DMMB assay was modified from previous reports to include a sequential digestion of individual samples. A volume of 300 μL of a solution containing 2 mM N-acetylcysteine and papain (0.13 units/100 μL) in PBS was added to an equal volume of diluted sample and incubated at 65°C for 2 h to release available GAG from the proteoglycan core protein. A volume of 150 μL of 20 mM iodoacetate in PBS was then added to each sample to stop the papain digestion. An aliquot of 250 μL of each sample was removed and assayed for total sulphated-GAG content ($A_{525\text{ nm}}$) after the addition of 2.5 mL DMMB reagent.

Samples were incubated for an additional 1 h after the addition of 100 μL of 0.25 units/mL chondroitinase ABC lyase (*Proteus vulgaris*, Sigma Chemical Co., St. Louis, Missouri) at 37°C to digest available CS in the sample. Chondroitinase

ABC also digests any HA and dermatan sulphate (DS) in the sample. However, since HA does not react with the DMMB reagent, and DS is believed to be a minor constituent of articular cartilage GAG, their influences on CS concentration should be minimal (2). A second aliquot of 300 μL of sample was removed and the absorbance determined as above with suitable blanks. The decrease in concentration from the total GAG value was considered to be the CS portion of the sample.

The remaining sample (300 μL) was again incubated at 37°C for 1 h after the addition of 50 μL of 0.1 units/mL keratanase (*Pseudomonas* sp, Sigma Chemical Co., St. Louis, Missouri) to digest available KS. The absorbance of this final digestion after the addition of DMMB reagent is associated with the cleavage of KS in the sample. The difference in absorbance after the addition of chondroitinase ABC and keratanase provided the concentration of CS and KS in each sample. The total sulphated-GAG concentration, minus both CS and KS, was attributed to either possible contamination from serum leakage into the joint or to the low amount of non-CS/non-KS GAG in the synovial fluid.

SAMPLE HYALURONATE ASSESSMENT

Synovial fluid HA concentrations were determined by a colorimetric assay after digestion by a microbial hyaluronidase (*Streptomyces hyalurolyticus*, Sigma Chemical Co., St. Louis, Missouri) (11,18). Briefly, standards of HA were prepared over a range of 7.81 to 500 $\mu\text{g}/\text{mL}$ in 0.4 M Na-acetate buffer (pH 5.0). Samples were diluted 1:5 in the same buffer. A 200 μL aliquot of samples and standards was digested with 50 turbidity-reducing units/mL hyaluronidase in a 60°C water bath for 4 h and then refrigerated overnight. A 0.04 M sodium periodate in 0.04 M sulfuric acid solution (250 μL) was added to each tube and incubated for 1 h in a 37°C water bath. A 3% solution of sodium arsenite in 0.5 M HCl (500 μL) was then added to each tube and allowed to sit until the disappearance of the iodine color (approximately 5 min). Thiobarbituric acid (4 mL of a 0.3% solution in 0.12 M HCl) was added to each tube and placed in a boiling water bath for 15 min. The

mixture was cooled to room temperature and an additional 4 mL of acid butanol (5% 12 N HCl in *n*-butanol) added to each tube for the development of the pink color. All tubes were centrifuged at $2000 \times g$ for 5 min, the top layer removed and the absorbance of the butanol layer read in a spectrophotometer ($A_{552 \text{ nm}}$). A plot of absorbance versus standard HA concentration was plotted and the values of each sample determined from the linear curve.

STATISTICAL ANALYSIS

Due to the skewed distribution, the comparisons of the mean synovial fluid sulphated-GAG, CS, KS, and HA concentrations and serum sulphated-GAG concentration were determined using a nonparametric Kruskal–Wallis ANOVA test followed by a post-hoc Dunn's multiple comparisons test, which corrects for the number of comparisons made. Correlations of total sulphated-GAG concentration to synovial fluid WBC count, total protein, HA, or serum sulphated-GAG was determined by simple regression. $P < 0.05$ was considered significant.

RESULTS

There was a progressive decrease in absorbance within the first 4 min after the addition of the DMMB reagent that was associated with precipitation of the sulphated-GAG/DMMB complex. To ensure the consistency of the reported values, the absorbance of all standards and samples was read within 30 s after the addition of the DMMB reagent.

As demonstrated by the standard curves, the complex of DMMB-CS resulted in a greater absorbance than the DMMB-KS complex. Since CS is the major GAG of the articular cartilage and synovial membrane, the total sulphated-GAG concentration was determined from the C6S standard curve. At the concentration extremes (i.e., $< 5.0 \mu\text{g/mL}$ and $> 50 \mu\text{g/mL}$), both the DMMB-CS and DMMB-KS absorbance become nonlinear. Therefore, values of sulphated-GAG concentration were only determined from the linear portion of the curve. The specificity of the DMMB dye for only sulphated-GAGs is demonstrated by the absence of DMMB-complex

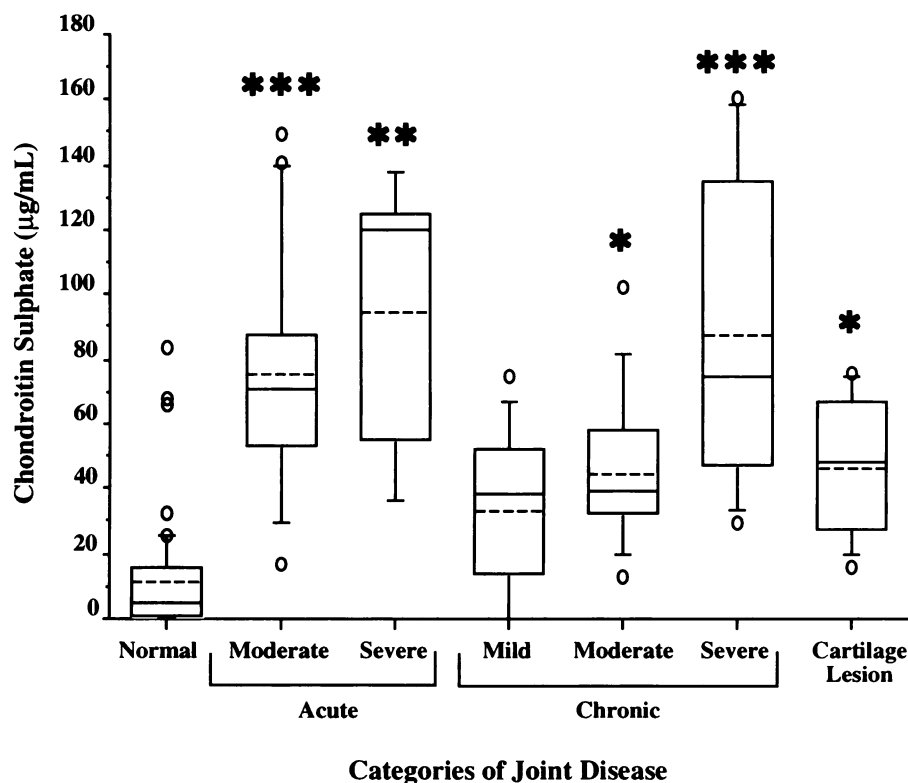


Fig. 2. Box plot of synovial fluid chondroitin sulphate (CS) concentration measured by DMMB assay and grouped according to joint disease status (Table I). Mean of values represented by dashed bar. Median of values represented by solid bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

formation with HA. Sulphated-GAG concentrations (mean \pm SEM) were elevated in horses with moderate acute ($106.4 \mu\text{g/mL} \pm 11.7 \mu\text{g/mL}$), severe acute ($159.0 \mu\text{g/mL} \pm 47.7 \mu\text{g/mL}$), moderate chronic ($78.3 \mu\text{g/mL} \pm 9.4 \mu\text{g/mL}$), and severe chronic ($122.9 \mu\text{g/mL} \pm 22.5 \mu\text{g/mL}$) joint pathology and in horses with detectable cartilaginous arthroscopic lesions ($67.2 \mu\text{g/mL} \pm 11.6 \mu\text{g/mL}$), but without synovitis or radiographic abnormalities as compared with synovial fluid total sulphated-GAG of normal horses ($25.6 \mu\text{g/mL} \pm 2.6 \mu\text{g/mL}$) (Fig. 1). Synovial fluid CS concentration (mean \pm SEM) was significantly greater when compared with normal joint synovial fluid ($12.4 \mu\text{g/mL} \pm 2.7 \mu\text{g/mL}$), in joints with moderate acute ($74.9 \mu\text{g/mL} \pm 7.7 \mu\text{g/mL}$), severe acute ($94.8 \mu\text{g/mL} \pm 19.8 \mu\text{g/mL}$), moderate chronic ($45.5 \mu\text{g/mL} \pm 7.8 \mu\text{g/mL}$), and severe chronic ($87.9 \mu\text{g/mL} \pm 17.7 \mu\text{g/mL}$) joint pathology and in horses with detectable cartilaginous arthroscopic lesions ($47.9 \mu\text{g/mL} \pm 7.5 \mu\text{g/mL}$), but without synovitis or radiographic abnormalities (Fig. 2). Synovial fluid

KS concentration (mean \pm SEM) was significantly greater than normal joint synovial fluid ($7.6 \mu\text{g/mL} \pm 1.2 \mu\text{g/mL}$) for joints with moderate chronic ($25.2 \mu\text{g/mL} \pm 5.1 \mu\text{g/mL}$) and severe chronic ($30.3 \mu\text{g/mL} \pm 5.7 \mu\text{g/mL}$) joint disease only (Fig. 3). Concentrations of non-CS/non-KS sulphated-GAGs (mean \pm SEM) not digested by chondroitinase ABC or keratanase were significantly greater in the synovial fluid of horses with moderate acute ($32.6 \mu\text{g/mL} \pm 5.8 \mu\text{g/mL}$) joint disease only (Fig. 4).

There were no significant differences in the concentrations of serum sulphated-GAGs (mean \pm SEM) in any category of inflammation studied as compared with normal ($157.0 \mu\text{g/mL} \pm 11.05 \mu\text{g/mL}$). In addition, there was no correlation between the serum and synovial fluid sulphated-GAGs concentrations. There was a highly significant, but low correlation ($P = 0.0001$) between synovial fluid sulphated-GAG concentration and synovial fluid WBC count ($r = 0.38$) and between synovial fluid sulphated-GAG concentration and synovial fluid total protein ($r = 0.54$). The concentration of HA (mean \pm

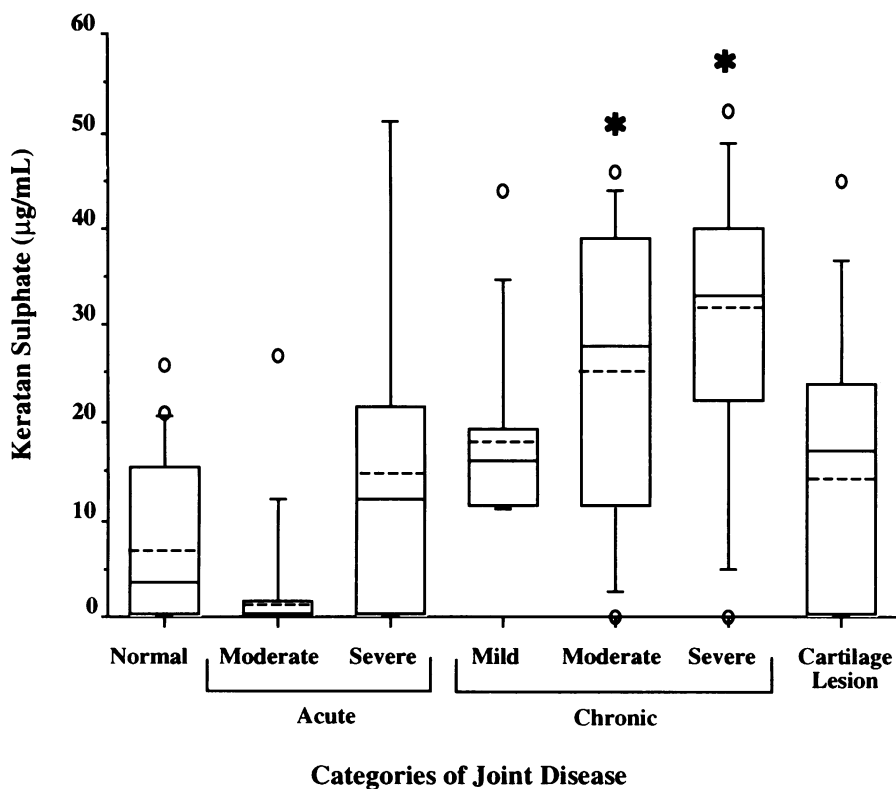


Fig. 3. Box plot of synovial fluid keratan sulphate (KS) concentration measured by DMMB assay and grouped according to joint disease status (Table I). Mean of values represented by dashed bar. Median of values represented by solid bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

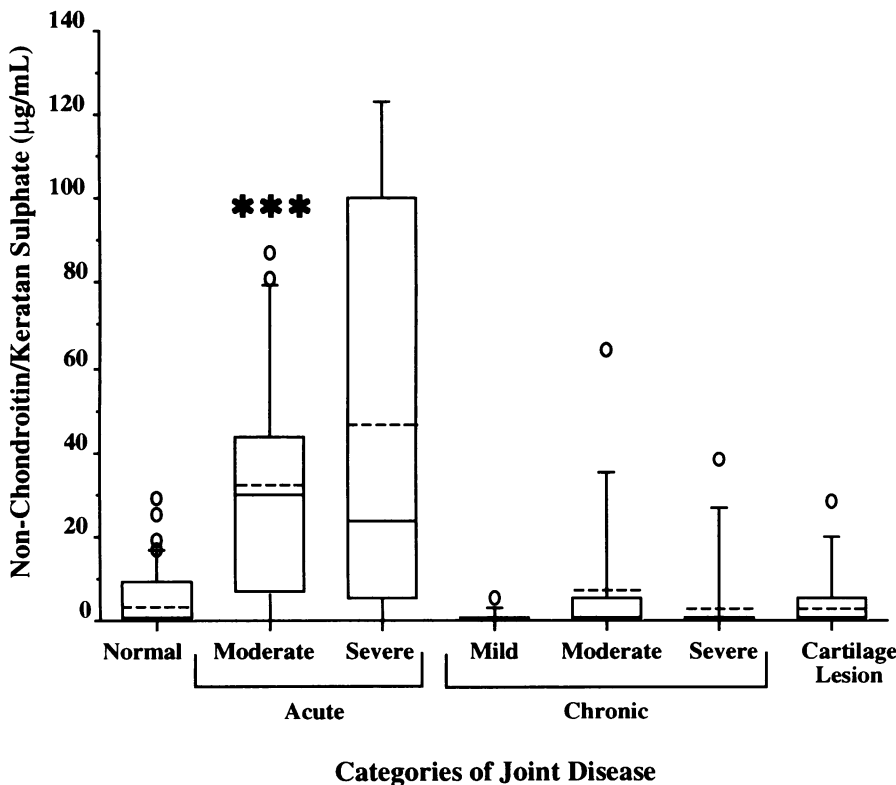


Fig. 4. Box plot of synovial fluid nonchondroitin/keratan sulphate glycosaminoglycan (non-CS/KS GAG) concentration measured by DMMB assay and grouped according to joint disease status (Table I). Mean of values represented by dashed bar. Median of values represented by solid bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

SEM) was not significantly different in any category of inflammation studied from normal ($260.7 \mu\text{g/mL} \pm 32.0 \mu\text{g/mL}$), and there was no correlation between synovial fluid sulphated-GAG concentration and synovial fluid HA concentration (Fig. 5).

DISCUSSION

The results of this study complement previous reports of increases in sulphated-GAG concentration in synovial fluid of patients with chronic joint disease (osteoarthritis) and various other arthritides, but increase the number and more fully delineate the range of clinical conditions seen with both acute and chronic joint disease in the horse (1–6). The large increase in synovial fluid sulphated-GAG concentration and CS in moderate and severe acute, and in moderate and severe chronic, joint disease may reflect alterations in the synthetic or degradative process of both the articular cartilage and synovial membrane since CS is a ground substance GAG common to both tissues. Keratan sulphate has a more limited distribution and, within synovial tissues, is primarily found only within the articular cartilage matrix, particularly in the deep zone. Therefore, the significantly high concentration of KS in the moderate and severe chronic joint disease groups may reflect erosion of the articular cartilage and exposure of the deeper cartilage layers. This agrees with previous reports of increased synovial fluid KS concentrations as measured by enzyme-linked immunosorbent assays (ELISA) in horses with experimentally created osteochondral defects and in horses with methylprednisolone acetate treated joints (15,16). To differentiate further the source of GAG within the synovial fluid, studies to detect the presence of proteins specific to articular cartilage, such as cartilage oligomeric matrix protein (COMP), may be necessary (9).

The high concentration of CS in the synovial fluid of all joint disease groups (excluding mild chronic joint disease) confirms that CS is the major contributor to the overall increase in synovial fluid total sulphated-GAG concentration. This may be associated with the greater percentage of CS (as compared with KS) in both the synovial membrane and superficial

articular cartilage matrix (2,7). Alternatively, cleavage of the larger CS chain moiety from the proteoglycan core protein may be required for subsequent KS cleavage due to possible CS steric protection of the smaller KS chains. Once cleaved, KS chains would be expected to more rapidly diffuse from the cartilage matrix due to their smaller size. Consequently, the increased concentration of KS in moderate and severe chronic joint disease likely reflects significant matrix destruction.

A significant finding of this study, however, is the significant increase in synovial fluid CS concentration in horses with arthroscopically visible cartilage lesions, but without concurrent synovitis or radiographic lesions. Since these horses lacked a clinically and clinicopathologically detectable synovitis, the contribution of synovial fluid CS concentration to total sulphated-GAG concentration may result solely from occult cartilage destruction. The importance of this finding may suggest the use of the DMMB assay as a possible method of detecting radiographically silent cartilaginous lesions prior to, or as an adjunct to, arthroscopic examination.

The highly significant correlation between synovial fluid total protein and sulphated-GAG concentration was expected. Inflammation of the synovial membrane is associated with increased blood flow and capillary permeability, thus allowing influx of circulating neutrophils and plasma proteins into the joint. Sulphated plasma components released into the synovial fluid during the inflammatory response may interfere with, and falsely elevate, the total sulphated-GAG concentration in synovial fluid (5). However, the use of specific glycosidases in this study to digest CS and KS from the total sample conclusively shows that the largest contribution to total sulphated-GAG concentrations is from sulphated-GAGs rather than from sulfated plasma proteins.

The significant correlation between synovial fluid sulphated-GAG concentration and synovial fluid WBC may be indicative of neutrophil migration into the inflamed joint and the subsequent release of cytokines, proteolytic enzymes, and eicosanoids that contribute to both articular cartilage

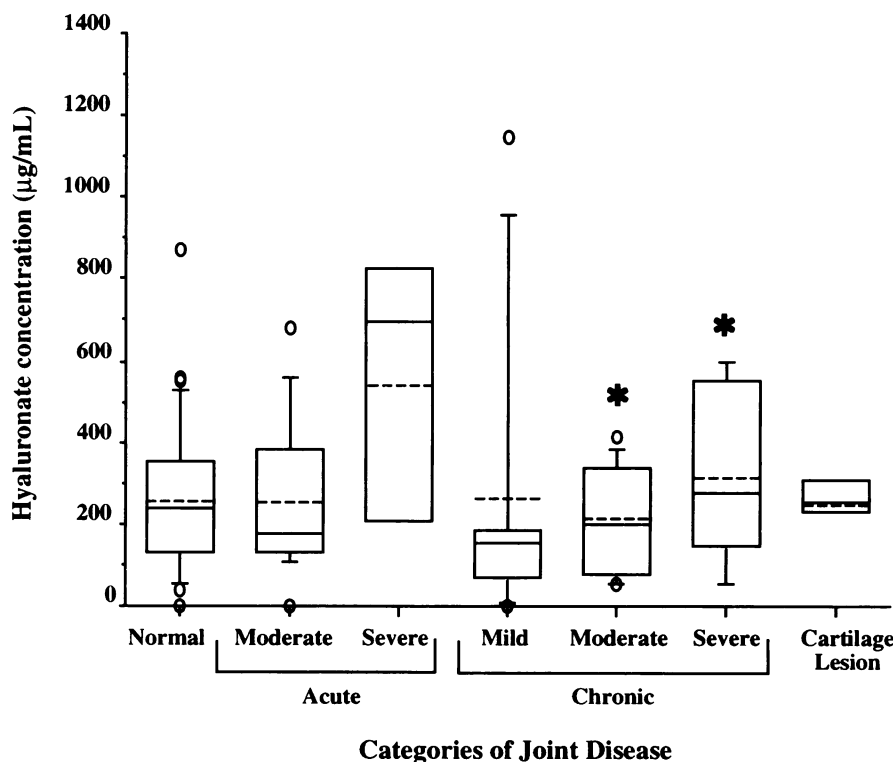


Fig. 5. Box plot of synovial fluid hyaluronate (HA) concentration grouped according to joint disease status (Table I). Mean of values represented by dashed bar. Median of values represented by solid bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

matrix and synovial membrane ground substance alterations. Activation of both synoviocytes and chondrocytes by these inflammatory mediators likely results in both an increase in chondrocyte synthetic activity and an increase in degradative products. The primary destructive mediators of articular cartilage and synovial membrane are the neutral metalloproteinases (stromelysin, collagenase, elastase) that degrade the proteoglycan core protein near its attachment site to hyaluronate. Cleaved proteoglycan fragments with attached GAG side chains may diffuse out of the synovial tissue matrix into the synovial fluid. Similarly, an increase in GAG synthesis in response to the inflammatory stimulus, without the ability of the GAG to be retained in the matrix, may also result in increased synovial fluid sulphated-GAG concentrations.

The mean concentration of HA detected in the synovial fluid of normal horses by this laboratory was much less than that reported previously in equine synovial fluid by the same method of analysis, but agreed with the mean concentration of synovial fluid HA as determined by

radioassay (11,19). The lack of difference detected among the joint disease groups is consistent with previous reports and may be a result of a dilutional effect associated with effusion into the joint during inflammation. Alternatively, a breakdown of released HA in pathological joints by hyaluronidases or superoxide radicals may decrease the function of synovial fluid HA without decreasing the total concentration of HA. This may account for the lack of association between synovial fluid HA concentration and mucin precipitate test results. The lack of correlation between synovial fluid HA concentration and sulphated-GAG concentration may be a reflection of the difference in size of articular cartilage CS or KS chains versus HA chains.

This study further explores the use of the DMMB assay as a possible marker of equine joint pathology. Ultimately the best marker or panel of markers of equine joint pathology would be detectable within the serum, since venipuncture is relatively less invasive than arthrocentesis. Due to the lack of detectable differences in serum sulphated-GAG in this work, this would appear to be a significant

limitation of the DMMB assay. The use of immunological markers may prove more beneficial in this regard since these tests are many times more sensitive and specific and may potentially provide additional information on joint "health" (i.e., degradation or repair) (20,21). However, immunoassays specifically designed for equine proteoglycan or collagen fragments are only available for KS, and this assay is not available on a commercial basis. Until these assays are proven for equine samples, the addition of the DMMB assay to routine synovial fluid analysis may be beneficial, particularly in detecting radiographically silent cartilaginous lesions prior to arthroscopic surgery.

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