Assessment of three variations of the 1,9-dimethylmethylene blue assay for measurement of sulfated glycosaminoglycan concentrations in equine synovial fluid

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Objective—To determine whether 3 variations of the 1,9-dimethylmethylene blue (DMMB) assay yield comparable results when measuring sulfated glycosaminoglycan (sGAG) concentrations in equine synovial fluid (SF).

Sample Population—25 samples of SF collected from affected joints of 13 horses and 13 samples of SF collected from nonaffected (control) joints of 4 horses.

Procedure—Sulfated glycosaminoglycan concentrations were measured by the direct spectrophotometric (ie, Farndale), microplate, and indirect DMMB assays in samples of SF collected from normal and affected joints and in samples digested with nucleases, papain, and hyaluronidase.

Results—All 3 assays reacted similarly to standard solutions of sGAGs and digestion of SF samples with nucleases, papain, and hyaluronidase. Nucleic acids were not important interfering substances, and papain and hyaluronidase could not be used interchangeably to digest SF. All 3 assays proved to have satisfactory precision (SD < 10%), but each DMMB assay resulted in significantly different measures of sGAG in equine SF.

Conclusions and Clinical Relevance—Samples of SF should be digested with papain or hyaluronidase prior to measurement via DMMB assay. Researchers currently are unable to compare clinical information when variations of the DMMB assay are used, because each DMMB assay yields substantially different sGAG concentrations in SF. Of the 3 assays examined here, we recommend use of the direct spectrophotometric DMMB assay. (*Am J Vet Res* 2003;64:900–906)

Osteoarthritis (OA), also referred to as degenerative joint disease, is a group of disorders characterized by alterations in metabolism of **articular cartilage (AC)** that leads to physical degeneration of the cartilage accompanied by changes in the bones and soft tissues of affected joints.[1-3] The study of OA is important in veterinary medicine, because joint injury and joint disease are major causes of lameness in athletic horses. In turn, lameness is the most important cause of wastage in these horses.[1,4-6]

The AC comprises chondrocytes embedded in an extracellular matrix of collagen, **proteoglycans (PGs)**, noncollagenous proteins, and water. The compressive stiffness of the extracellular matrix afforded by the high water content permits AC to withstand deformation during joint articulation.[7,8] The PGs, predominantly in the form of aggrecan, represent the major component of the AC matrix (10% of cartilage on a dry-weight basis) and are essential for normal function of AC.[9] Alterations in PG structure and concentration or changes in compressive stiffness of AC contribute to cartilage degeneration during chronic inflammation and OA. Proteoglycans are also major targets of degradative enzymes released from inflammatory cells or chondrocytes during OA.[5,8,10] Moreover, cytokines (such as interleukin-1 and tumor necrosis factor-α) are released during synovitis that accompanies joint disease. This leads to chondrocyte-mediated destruction of the AC through upregulation of neutral metalloproteinases that degrade the core protein of aggrecan.[2] Metalloproteinases, such as stromelysin (ie, **matrix metalloproteinase [MMP]**-3) and collagenase (ie, MMP-1), are likely to play a major role in AC degradation.[1]

During normal turnover of cartilage, small amounts of low-molecular-weight PGs (ie, PG fragments) are released into the **synovial fluid (SF)**. In OA, higher molecular weight PG fragments, aggregates, and collagen breakdown products are released. These structures are then engulfed by chondrocytes or diffuse into the SF to ultimately reach the blood and urine.[3,9,11-14] Depletion of aggrecan from the cartilage matrix compromises the ability of AC to bear weight and further contributes to mechanical disruption of cartilage.[7]

Racehorses with large osteochondral injuries, such as intra-articular chip fractures, have a poor prognosis for future racing and a predisposition for development of OA.[3] Thus, early detection and treatment of OA is imperative, because structural alterations in AC are typically irreversible by the time radiographic changes of OA are evident.[2] Therefore, a noninvasive biochemical marker to monitor AC destruction in vivo prior to

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radiographic detection of degradative changes would be invaluable. Armed with this marker, clinicians could assess severity of disease, monitor response to treatment, potentially slow the development of OA, and, thus, safeguard future athletic performance.[2,13] Potential markers include, but are not limited to, total **sulfated glycosaminaglycon (sGAG)**, chondroitin sulfate, keratan sulfate, link protein, cartilage oligomeric protein, and type-II collagen peptide.[15]

A multitude of research groups use the **1,9-dimethylmethylene blue (DMMB)** assay as part of the routine analysis of SF. It also has been used in the search for molecular markers. At first glance, this assay appears quite simple; positively charged DMMB dye is added to a solution containing negatively charged sGAGs, and the absorbance of the resultant sGAG-dye complexes is measured at a wavelength of 525 to 535 nm.[16-18] Unfortunately, the simplicity of the assay is deceptive. One direct spectrophotometric method (the Farndale method), which is widely accepted as a simple, reliable, reproducible, and sensitive assay for measuring sGAG concentrations of tissues and fluids,[16,18,19] is problematic, because instability of the sGAG-dye complexes is characterized by a slow, progressive decrease in absorbance at a wavelength of 535 nm during the first 10 minutes of the assay.[18] An assay that allegedly alleviates this problem was provided by Müller and Hanschke.[20] They developed an indirect spectrophotometric assay for sGAG concentrations that involved adding DMMB dye to the sGAG solution and allowing the sGAG-dye complexes to precipitate at room temperature for 30 minutes. The sGAG concentration is determined by measuring the change in absorbance of the DMMB solution at a wavelength of 595 nm.[20] A microplate method also was developed that involves the use of small volumes of dye and sample in a 96-well microplate; results are determined by measuring absorbance at a wavelength of 540 nm.[21]

In addition to instability of dye-sGAG complexes, there is potential interference by polyanionic molecules (eg, nucleic acids or hyaluronic acid) when measuring sGAG contents in biological fluids by use of the DMMB assay. Finally, it has been found that SF samples must be enzymatically digested prior to performing the DMMB assay to prevent formation of a blue, stringy precipitate that renders spectrophotometric analysis impossible. It has been reported[16] that papain or hyaluronidase may be used for this purpose. Because of inherent differences in the mechanism of action of these 2 enzymes, this suggestion is being challenged.

Interestingly, in our experience, the measurement of sGAG concentrations in SF by use of 3 common versions (ie, direct, microplate, and indirect) of the DMMB assay has yielded substantial differences in estimates of sGAG content in samples of SF. Because the measurement of sGAG concentrations in equine SF is a standard procedure performed by most researchers who study joint injury and joint disease in horses, the study reported here was designed to critically compare these 3 DMMB assays in their ability to measure sGAG concentrations in samples of equine SF. Although we did not expect to obtain absolutely identical values for sGAG contents by use of each of the 3 assays, it was anticipated that there would be a relationship between them, and there would not be statistical differences among results.

Materials and Methods

Collection and preparation of SF samples—Samples of SF were collected from affected and nonaffected (control) joints of horses. Synovial fluid from nonaffected joints was obtained from horses that had been euthanatized for reasons other than musculoskeletal conditions. Samples were collected aseptically into EDTA-coated, 10-mL evacuated tubes. Samples were collected during necropsy, within 8 hours after horses were euthanatized. After aseptic collection of SF, nonaffected joints were dissected to ensure that there was no macroscopic evidence of joint disease. Samples of SF from affected joints were collected from horses undergoing arthroscopic surgery for various types of pathologic joint conditions.

All samples were centrifuged (20,000 × *g* for 30 minutes at 4°C) as soon as possible after collection. The SF supernatants were decanted and stored in aliquots at –80°C until time of analysis (approx 9 to 12 months). Samples are stable for several years under these conditions.[3,10] In total, 25 samples of SF were collected from affected joints of 14 horses, whereas 13 samples of SF were collected from nonaffected joints of 4 horses. Diagnoses were made by the attending surgeon at the time of arthroscopic surgery or by the attending pathologist during necropsy. Unless indicated otherwise, all SF samples were digested prior to analysis by incubation with an equal volume of papain[a] (1 mg/mL) in papain digestion buffer (0.69 g of sodium phosphate monobasic, 0.0326 g of *N*-acetyl cysteine, and 0.076 g of EDTA tetrasodium salt in a volume of 100 mL [pH, 6.5]) at 65°C for 3 hours.

Preparation of dye—Dye was prepared in accordance with the method of Farndale et al.[17] Five milliliters of 95% ethanol was added to 16 mg of DMMB,[b] and the mixture was allowed to incubate for 30 minutes at 20°C. Two milliliters of formate buffer (1.54 mL of formic acid and 2.94 mL of 5 N NaOH in a volume of 500 mL [pH, 3.5]) was added, and water then was added to achieve a final volume of 1 L. The resultant dye, which had an approximate absorbancy of 0.34 at a wavelength of 535 nm, was stored protected from light at room temperature (20°C) for up to 2 months. Although some researchers store the dye at 4°C, it was reported in 1 study[18] that the color of the dye fades at such a low temperature.

Direct spectrophotometric DMMB assay—Samples of equine SF that had been digested with papain (or other enzymes, when necessary) were typically diluted 1:5 with water (ie, 1 part synovial fluid added to 4 parts water). A 250-µL volume of digested synovial fluid was placed in 1.5-mL polypropylene microcentrifuge tubes.[c] Immediately before measuring the absorbance at 525 nm on a spectrophotometer,[d] the samples were transferred from the polypropylene tubes to disposable spectrophotometry cuvettes, and 2.5 mL of dye was added. Samples were gently mixed by inverting each cuvette 2 or 3 times. The sGAG content of each SF sample was determined by comparison with a standard curve that was prepared with each assay. The standard curve was created by preparing solutions containing 0 to 25 µg of **chondroitin sulfate C** (**CSC**[e]; stock solution contained 100 µg/mL of water) in a final volume of 250 µL. All standard solutions and samples were prepared and assayed in triplicate, and appropriate blank samples were included in each assay.

Microplate DMMB assay—A microplate DMMB assay, based on the method of Goldberg and Kolibas[21] and modified by another investigator,[f] was performed. Samples of digested

synovial fluid were diluted with water (1:10 or 1:20). Aliquots (50 µL each) of diluted SF samples were applied to wells of a 96-well flat-bottom plate.[g] Immediately before measuring absorbance at 540 nm on a 96-well microplate reader,[h] 200 µL of DMMB dye was added to each well. A standard curve was prepared with each set of samples by use of aliquots (50 µL) of serial solutions of CSC (containing 0 to 10 µg of CSC) in wells of the 96-well flat-bottom plate. All standard solutions and samples were prepared and assayed in triplicate, and appropriate blank samples were included in each assay.

Indirect spectrophotometric DMMB assay—In 1.5-mL polypropylene microcentrifuge tubes, SF samples were diluted (typically 1:5) with water to achieve a final volume of 100 µL. Dye (1.25 mL) was added to each polypropylene tube, and the resultant solution was mixed thoroughly in a vortexer. After incubation at 20°C for 30 minutes, the tubes were centrifuged at 10,000 × *g* for 15 minutes. An aliquot (200 µL) of supernatant from each sample and standard solution were pipetted into a 96-well flat-bottom plate. Absorbance of the supernatant was measured against the appropriate blank samples at 600 nm on a 96-well microplate reader.[i] The sGAG content of each SF sample was determined by comparison with a standard curve that was prepared with each assay. Standard curves were created by preparing solutions containing 0 to 10 µg of CSC. All standard solutions and samples were prepared and assayed in triplicate, and appropriate blank samples were included in each assay.

Reaction of DMMB with standard solutions of control sGAGs—The 3 DMMB assays were evaluated by comparing their ability to react with a series of standard solutions of sGAGs including CSC, **chondroitin sulfate A (CSA)**,[j] and **keratan sulfate (KS)**.[k] Stock solutions (100 µg/mL of water) were prepared for each of the 3 sGAGs, and 0 to 25 µg of each sGAG was assayed by all 3 methods to determine linear range of the assays. All standard solutions and samples were prepared and assayed in triplicate, and appropriate blank samples were included in each assay.

Evaluation of interference of nucleic acids—Two samples of SF were obtained from the right and left tibiotarsal joints of a yearling Standardbred horse with bilateral osteochondritis dissecans; each sample was divided into 2 aliquots. One aliquot of each sample was digested with nucleases by adding 100 µL of a stock solution (1 mg/mL) of DNase[l] and 100 µL of a stock solution (0.1 mg/mL) of RNase[m] per 1.5 mL of SF; the mixture was then incubated at 25°C for 30 to 60 minutes. This digestion was followed by routine digestion with papain, as described previously. The second aliquot of SF was digested with papain only. The sGAG content of the digests was measured by use of each of the 3 DMMB methods. The sGAG concentrations for SF digested by the nucleases were compared with the sGAG concentrations for SF samples that were not digested by the nuclease. Each sample was assayed 3 times in triplicate by each method.

Enzymatic digestion of SF samples—Two samples of equine SF were obtained from the right and left tibiotarsal joints of a yearling Standardbred horse with bilateral osteochondritis dissecans; each sample was divided into 4 aliquots. One aliquot of each sample was digested by incubation with 10 units of *Streptomyces* hyaluronidase[n]/mL at 34°C for 1 hour, whereas the second aliquot was digested by incubation with an equal volume of papain (1 mg of papain/mL of SF in papain digestion buffer) at 65°C for 3 hours. The third aliquot was initially digested by incubation with *Streptomyces* hyaluronidase for 1 hour at 34°C followed by digestion with papain (as described previously), and the fourth aliquot of each sample was digested by incubation with 100 µL of a solution of hyaluronidase (100 U/mL) at 37°C for 1 hour followed by incubation at 65°C for an additional 3 hours (no papain digestion). Resultant digests were measured (in triplicate) by use of each of the 3 DMMB assays 3 times to determine sGAG concentrations.

These samples were then examined by use of SDS-PAGE followed by a stringent staining regimen that used Alcian blue and silver stain. Briefly, a 4–20% polyacrylamide gradient separating gel was prepared by use of a commercial system.[o] The gradient gel was created manually by layering 1 mL (from bottom to top) of 20, 15, 10, and 4% acrylamide solutions. Partial mixing of the layers prior to polymerization linearized the gradient. Acrylamide solutions were prepared in 1.0 mL of 1.5M Tris-HCl (pH, 8.8), 40 µL of 10% SDS, 20 mL of 10% **ammonium persulphate (APS)**, and 2 µL of **tetramethylethylenediamine (TEMED)**. The gradient gel was prepared in conjunction with a 3.6% stacking gel that contained 3.1 mL of H_2O, 1.25 mL of 0.5M Tris HCl (pH, 6.8), 600 µL of acrylamide-bis (30% T [ie, total monomer concentration], 2.67% C [ie, crosslinker]), 50 µL of 10% SDS, 30 mL of 10% APS, and 5 µL of TEMED. Equal volumes of sample, each of which contained 4 mg of sGAG, were heated at 100°C for 10 minutes and then loaded in 4× sample buffer. Ten microliters of prestained marker[p] were assayed with each gel. The SDS-PAGE was performed at a constant current of 40 mA for approximately 45 minutes or until the dye front had migrated off the end of the gel. The 1× running buffer was prepared from a 5× stock solution (7.5 g of Tris base, 36 g of glycine, and 3 g of SDS in a final volume of 500 mL [pH, 8.3]). Gels were stained sequentially with Alcian blue dye (to color sGAGs) and silver stain (to bind proteins).[22]

Use of DMMB assays on clinical samples—The direct spectrophotometric method and microplate DMMB assay were used to measure sGAG concentrations in 34 samples of SF collected from affected and nonaffected joints of horses. All samples were digested with papain prior to analysis.

Statistical analysis—Data were analyzed for normality by use of a univariate analysis. After logarithmic transformation, data were subjected to analysis by use of a mixed-model ANOVA to test for differences attributable to enzymes (nucleases and papain or hyaluronidase) or assays (direct spectrophotometric method, microplate DMMB assay, and indirect DMMB assay). Adjusted mean differences were not considered significant when the 95% confidence intervals of the means contained a value of 0. Values of $P < 0.05$ were considered significant.[q]

Results

Reaction of DMMB with various control sGAGs—Standard curves for KS, CSC, and CSA were determined by use of the direct spectrophotometric DMMB assay (**Fig 1**). The standard curves for each of the 3 sGAGs differed; CSC consistently resulted in the most intense metachromasia (ie, had the largest slope), whereas KS invariably had the smallest slope, and CSA had an intermediate slope. Similar curves and patterns were observed for the microplate and indirect DMMB assays. The curve for the direct spectrophotometric method was linear between 0 and 25 µg of sGAG (ie, CSC, CSA, and KS). The curve for the microplate DMMB assay was linear between 0 and 2.0 µg of sGAG, whereas the curve for the indirect DMMB assay was linear between 1 and 10 µg of sGAG. All standard curves had strong linearity within the given range (R^2, > 0.99).

Interference of nucleic acids—We did not detect significant (P = 0.19) differences in sGAG concentrations between SF samples that had been digested with DNase and RNase and those that had not been digested with the nucleases (**Fig 2**). This finding was consistent for each of the 3 DMMB assays.

Enzymatic digestion of SF—The SF samples digested with papain alone yielded significantly higher concentrations of sGAGs than the same samples digested with hyaluronidase alone (P = 0.029) or with both enzymes sequentially (P = 0.043). Although SF samples digested with both enzymes sequentially typically yielded intermediate results, there was not a significant (P = 0.391) difference between samples digested with hyaluronidase alone or with both enzymes (**Fig 3**). This finding was consistent for all 3 DMMB assays. Samples digested with hyaluronidase at 34°C for 1 hour and then 65°C for 3 hours had a stringy precipitate that persisted in the solution. Therefore, those data were excluded from all analyses.

Synovial fluid samples were separated on a 4–20% linear gel by use of SDS-PAGE; this was followed by use of Alcian blue stain for sGAGs and silver stain for protein (results not shown). Undigested SF, hyaluronidase-digested SF, and papain-digested SF each yielded high molecular weight bands (> 250 kd) stained with Alcian blue. Conversely, SF samples digested sequentially with hyaluronidase and then papain did not yield any bands stained with Alcian blue. After staining the same gel with silver stain, a single band of approximately 60 kd was identified in the undigested and hyaluronidase-digested SF samples. Interestingly, the papain-digested sample and the sample digested sequentially with hyaluronidase and then papain resulted in similar, complex banding patterns. The primary difference between these samples was the lack of high-molecular-weight bands in the SF samples digested with both enzymes sequentially.

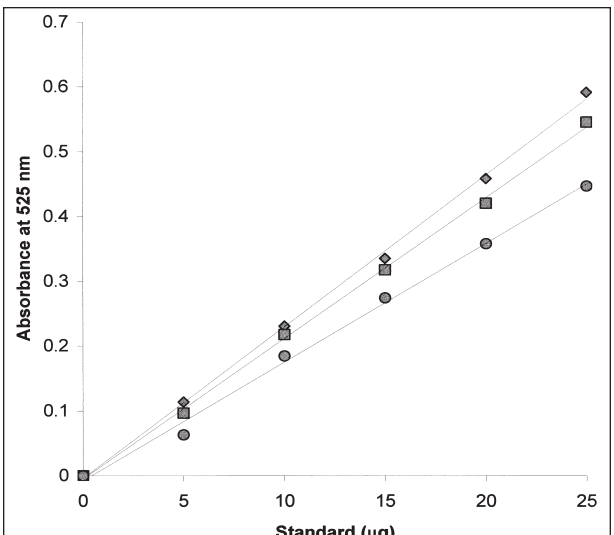

Figure 1—Curves for standard solutions of the sulfated glycosaminoglycans (sGAGs) chondroitin sulfate C (diamond), chondroitin sulfate A (square), and keratan sulfate (circle) measured by use of the direct spectrophotometric method of the 1,9-dimethylmethylene blue (DMMB) assay. The R^2 values for CSC, CSA, and KS were 0.9988, 0.9988, and 0.9957, respectively.

Comparison of the 3 assays—Although all 3 DMMB assays yielded similar patterns in the measurement of sGAGs in SF samples digested with nucleases, papain, and hyaluronidase, the absolute amounts of sGAGs measured by each of the 3 assays differed. The direct spectrophotometric assay consistently yielded the lowest estimate of sGAG concentrations in equine SF samples, compared with results for the other 2 assay methods (**Fig 2 and 3**). The microplate DMMB assay

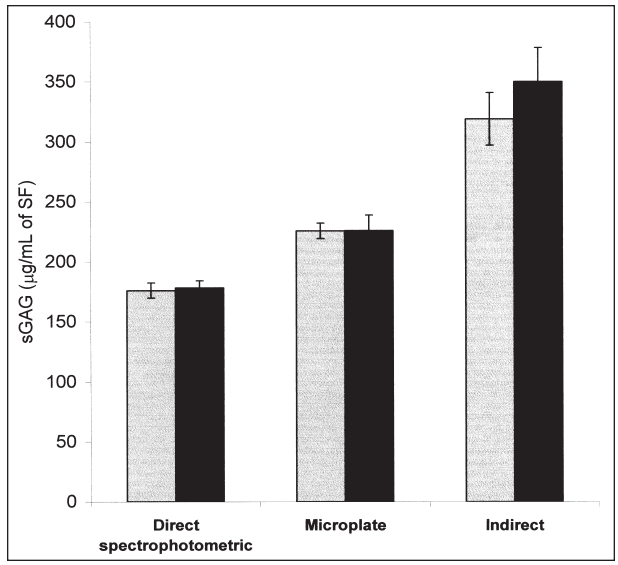

Figure 2—Effect of nuclease digestion on sGAG concentrations in synovial fluid (SF) obtained from joints of a horse with osteochondritis dissecans measured by use of 3 DMMB assays. Mean ± SD sGAG concentrations were measured by the direct spectrophotometric method, microplate DMMB assay, and indirect DMMB assay in samples digested (gray bars) or not digested (black bars) by incubation of SF with DNase and RNase.

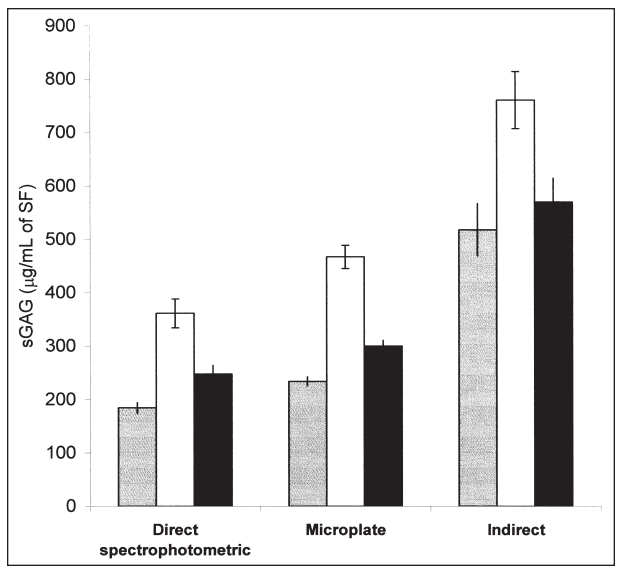

Figure 3—Effect of enzymatic digestion on sGAG concentrations in SF obtained from joints of a horse with osteochondritis dissecans. Each SF sample was divided into aliquots; aliquots were digested by incubation with hyaluronidase (gray bar), papain (white bar), or hyaluronidase followed by papain (black bar), and mean ± SD sGAG concentrations were then measured by use of the direct spectrophotometric method, microplate DMMB assay, and indirect DMMB assay.

yielded intermediate results, whereas the indirect DMMB assay yielded the highest values for sGAG concentrations. Values for the 3 DMMB assays differed significantly ($P < 0.001$), as determined by the use of pairwise tests.

Use of DMMB assays on clinical samples—The sGAG concentrations of 34 samples of SF obtained from horses were determined by use of the direct spectrophotometric method and microplate DMMB assay. The sGAG concentrations obtained by the microplate DMMB assay were significantly ($P = 0.042$) higher than concentrations obtained by the direct spectrophotometric method (**Fig 4 and 5**).

The sGAG content in nonaffected joints was significantly ($P < 0.001$) higher than that in affected joints, although a few samples did not follow this pattern (Fig 4 and 5). Comparison of the sGAG concentrations between joints revealed that although the sample size was too limited to perform statistical analyses, the pattern did not indicate differences in sGAG content between affected and nonaffected joints.

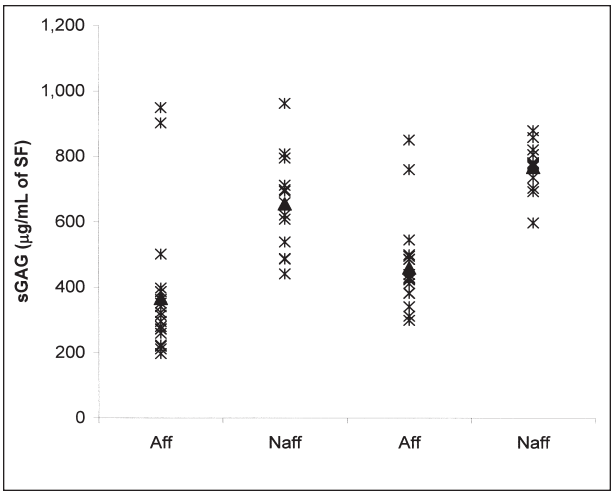

Figure 4—Concentration of sGAG in SF samples obtained from affected (Aff) and nonaffected (Naff) joints of horses and measured by use of the direct spectrophotometric method and microplate DMMB assay. Each asterisk represents the concentration in an individual joint, whereas a triangle represents the mean sGAG concentration for all joints in a category.

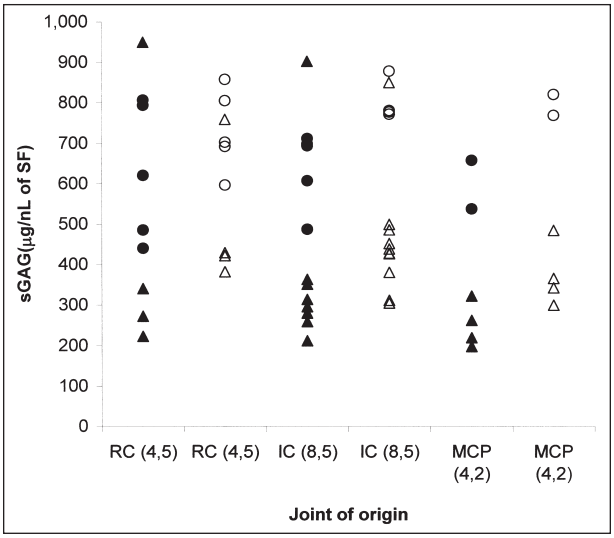

Figure 5—Sulfated GAG concentrations in SF samples obtained from various joints of horses and measured by use of the direct spectrophotometric method (black symbols) and the microplate DMMB assay (white symbols). Samples were obtained from affected (triangles) and nonaffected (circles) joints of horses. Each symbol represents the concentration in 1 joint. Numbers in parentheses represent the number of SF samples obtained from affected and nonaffected joints, respectively. RC = Radiocarpal joint. IC = Intercarpal joint. MCP = Metacarpophalangeal joint.

Discussion

Regardless of the assay used, intensity of the metachromasia associated with CSC was consistently greater than that observed for CSA or KS. This confirms the findings of other research groups.[10,17-19,21,23] According to 1 study,[24] an equal response for all types of GAGs is required to quantitate samples that contain unknown types of GAGs. However, an investigator in another study[25] indicated that when the GAG composition of the samples is unknown, CSC serves as a good control sGAG. It has been suggested that the observed differences between control sGAGs reflects differences in charge density of the various types of GAGs and that both the carboxyl and sulfate moieties are important.[24] This is interesting, considering chondroitin sulfate contains uronic acid, whereas KS is a repeating disaccharide of D-galactose and *N*-acetyl D-glucosamine. Therefore, KC has 1 fewer ionizable groups than chondroitin sulfate that could react with DMMB dye.[26] If this were the only factor involved in the decrease in intensity of the metachromasia, then one would not expect a difference between CSC and CSA, because both possess the same number of carboxyl and sulfate groups. Perhaps the difference in the intensity of metachromasia between CSC and CSA is simply attributable to the inherent molecular heterogeneity of GAGs. Furthermore, commercial CSC and CSA are not pure (eg, CSC is 90% CSC and 10% CSA), and chondroitin sulfate chains vary in their sulfation. A single chain typically has stretches that are sulfated at the 4 position followed by stretches sulfated at the 6 position; however, there may be regions that are not sulfated or other regions that are sulfated at both the 4 and 6 positions.[27,28]

To our knowledge, the mechanism for the reaction between DMMB dye and sGAGs remains unclear. To understand the reactions of the various sGAGs with DMMB, this mechanism must be elucidated.

Consistent with other studies,[18,21] we found that nucleic acids do not interfere with the DMMB assay. To our knowledge, none of the other studies compared the effects of digesting samples of SF with nucleases followed by measurement of sGAGs. This is a direct means of determining the effect of endogenous nucleic acids, which may be a more accurate evaluation of the importance of nucleic acids than measuring the effect of exogenous administration of nucleic acids. Because SF typically has < 500 cells/mL[29] and most research groups centrifuge SF samples immediately after acquisition, it is not surprising that nucleic acids are not found in substantial concentrations in SF and, thus, do not interfere in sGAG assays.

Samples of SF must be enzymatically treated prior to addition of the DMMB dye or else a purple stringy material will precipitate. It is obvious, however, that papain-digested SF samples yielded a higher concentration of sGAGs than samples digested with *Streptomyces* hyaluronidase or samples sequentially digested with hyaluronidase and then papain. This contradicts recommendations of another investigator,[16] who stated that SF samples should be treated with papain or *Streptomyces* hyaluronidase. Our finding is not surprising considering the mechanism of the 2 enzymes. Papain is a protease that hydrolyses protein, and it has broad specificity for peptide bonds.[30] It is believed that papain releases sGAGs from the core protein in SF.[8] In contrast, *Streptomyces* hyaluronidase is a hyaluronoglucosidase that randomly hydrolyses 1,4-linkages between *N*-acetyl-β-D-glucosamine and D-glucuronate residues in hyaluronic acid.[30] In the study reported here, SF samples were successfully digested with hyaluronidase alone (ie, stringy precipitate did not form); therefore, papain is not the only enzyme capable of digestion of SF samples for analysis. Because sGAGs are not released from the core protein in hyaluronidase-digested samples (in contrast to their release in papain-digested samples), the persistence of tightly packed sGAGs may result in steric hindrance that does not enable DMMB molecules to bind fully to sGAGs along the length of the polymers. This theory is supported by the fact that sGAG concentrations were higher in papain-digested samples, compared with concentrations in hyaluronidase-digested samples. Thus, it is possible that higher sGAG concentrations are obtained in papain-digested samples because of sGAG release from the core protein, thereby exposing all polyanionic sites for binding with DMMB.

Theoretically, sequential digestion of SF samples with hyaluronidase and papain should have maximized the sGAG reaction with the DMMB dye. Interestingly, this phenomenon was not observed. However, it is possible that hyaluronic acid could have interfered with the DMMB assay in samples digested with papain alone, thereby resulting in spuriously increased sGAG concentrations. Therefore, it is possible that both hyaluronidase and papain digestion are required to measure sGAG concentrations in SF samples; hyaluronidase is essential to eliminate interference caused by hyaluronic acid, and papain is required to eliminate steric hindrance caused by sGAGs that are bound to the core protein. Although this theory is interesting, the banding patterns observed after separation of the samples by SDS-PAGE did not support this view. Specifically, samples digested sequentially with hyaluronidase and then papain did not appear to have any bands that stained with Alcian blue. It appeared that SF was digested differently during sequential enzymatic digestion, compared with samples digested with each enzyme individually, because we did not detect a recognizable correlation between banding patterns for the various samples.

All 3 DMMB assays proved to have satisfactory precision. Samples were assayed in triplicate 3 times; therefore, 9 measurements of sGAG concentrations were obtained for each SF sample by each assay, and the SD for each was < 10%. Nonetheless, it is not possible to conclude which of the assays is most accurate in measuring sGAG concentrations in SF, because a criterion-referenced standard assay for sGAGs does not exist. Initially, the fact that the indirect assay consistently estimated higher sGAG concentrations than either the direct spectrophotometric or microplate assays was surprising, because all 3 assays appeared to be based on the same principle (sGAG-dye interaction in proportion to the charge density). However, it is more accurate to suggest that the indirect assay does not measure metachromasia in the same manner as the direct spectrophotometric or microplate assays, because the sGAG-dye complexes are not being measured directly. Perhaps there are molecules other than hyaluronic acid or protein that exist in SF that bind to DMMB dye only after a period of prolonged contact (eg, 30 minutes at room temperature), resulting in spuriously increased measurement of sGAG concentrations. Such molecules could be electrolytes, urea, or sulfated plasma components, because SF is an ultrafiltrate of plasma.[29] The microplate DMMB assay is simply a scaled-down version of the direct spectrophotometric method; thus, it is surprising that they do not have better agreement of results. Our supposition that the indirect DMMB assay yields falsely high measures of sGAG content is supported by comparing sGAG concentrations obtained by other research groups.[8-10] Typical sGAG concentrations reported in those studies ranged from 25 to 250 μg/mL of SF, depending on the disease condition.

Concentrations of sGAGs in SF collected from nonaffected joints were higher than in affected joints. This finding contradicts a number of other studies[8-10] but is in agreement with the results of 1 study.[2] All SF samples collected from affected joints included in the study reported here were obtained immediately before arthroscopic surgery; therefore, lesions were chronic in nature. The lower concentrations of sGAGs in SF from affected joints, compared with concentrations in nonaffected joints, may have indicated that the cartilage was in an advanced stage of disease and characterized by loss of cartilage mass.[2] Thus, less PGs are extruded into the SF, because less PGs are available.

Alternatively, SF samples obtained from nonaffected joints may have been a poor representation of normal SF. Although gross evidence of joint disease was not observed during dissection of the nonaffected joints, 3 of the 4 horses included as control animals had chronic systemic illnesses and, therefore, were not representative of a healthy control population. Most research groups use horses that are euthanatized for reasons other than musculoskeletal conditions but that may be systemically ill. Ideally, researchers would use healthy horses without a recent history of lameness and that have not undergone arthroscopic surgery as a source of control samples; unfortunately, it is difficult to obtain large numbers of samples from those types of horses.

Problems inherent in the DMMB assay clearly remain unresolved; however, alternate techniques for accurate quantification of PGs remain equally problematic, and a criterion-referenced standard assay currently does not

exist. Regardless of its shortcomings, we believe that the DMMB assay remains unequivocally the best method currently available for researchers to use to estimate the sGAG content of SF and articular cartilage. More specifically, the direct spectrophotometric method appears to be the assay of choice for use by researchers to evaluate connective tissue. This recommendation is made on the basis of comparing results for sGAG concentrations measured in the study reported here with values of sGAG concentrations reported elsewhere,[8-10] and because precipitation of the sGAG-dye complexes does not appear as rapidly in the direct spectrophotometric method as during the microplate DMMB assay. It is our opinion that the cost of equipment and time commitment are approximately equal for all 3 assays and should not play an important role in selecting which method to use. Nonetheless, there is a need for a better method to estimate sGAG concentrations in equine SF if researchers are to share and compare meaningful data. The study reported here also documents the importance of having all researchers in this field of investigation use the same DMMB assay and an identical protocol (eg, use of enzymatic treatment).

[a]Papain, Sigma Chemical Co, St Louis, Mo.
[b]1,9-dimethylmethylene blue, Sigma-Aldrich Chemical Canada Ltd, Oakville, ON, Canada.
[c]Eppendorf polypropylene microcentrifuge tubes, 1.5 mL, Fisher Scientific, Ottawa, ON, Canada.
[d]SmartSpec 3000, Bio-Rad Laboratories, Hercules, Calif.
[e]Chondroitin sulfate C, Sigma Chemical Co, St Louis, Mo.
[f]Performed by Dr. Nancy Burton-Wurster, Baker Animal Health Institute, College of Veterinary Medicine, Cornell University, Ithaca, NY.
[g]Corning brand 96-well flat bottom plates, Fisher Scientific, Ottawa, ON, Canada.
[h]Titretek Multiscan MCC microplate reader with a 450-nm filter, Flow Laboratories, DuPont, Toronto, ON, Canada.
[i]Bio-Tek Instruments Inc, model EL311 (600-nm filter), Mandel Scientific, Guelph, ON, Canada.
[j]Chondroitin sulfate A, Sigma Chemical Co, St Louis, Mo.
[k]Keratan sulfate, Sigma Chemical Co, St Louis, Mo.
[l]DNase type I grade II from bovine pancreas, Roche Diagnostics, Mississauga, ON, Canada.
[m]RNase A, Roche Diagnostics, Mississauga, ON, Canada.
[n]*Streptomyces* hyaluronidase, Calbiochem, San Diego, Calif.
[o]BioRad mini-PROTEAN II electrophoresis system, Bio-Rad Laboratories, Hercules, Calif.
[p]BioRad precision pre-stained marker, Bio-Rad Laboratories, Hercules, Calif.
[q]*SAS/STAT software: changes and enhancements through release 6.12*. Cary, NC: SAS Institute Inc, 1997.

References

1. McIlwraith CW. General pathobiology of the joint and response to injury. In: McIlwraith CW, Trotter GW, eds. *Joint disease in the horse*. Philadelphia: WB Saunders Co, 1996;40–70.

2. Todhunter RJ, Fubini SL, Freeman KP, et al. Concentrations of keratan sulfate in plasma and synovial fluid from clinically normal horses and horses with joint disease. *J Am Vet Med Assoc* 1997;210:369–374.

3. Todhunter RJ, Yeager AE, Freeman KP, et al. Keratan sulfate as a marker of articular cartilage catabolism and joint treatment in ponies. *Am J Vet Res* 1993;54:1007–1016.

4. Brama PA, TeKoppele JM, Bank RA, et al. Influence of site and age on biochemical characteristics of the collagen network of equine articular cartilage. *Am J Vet Res* 1999;60:341–345.

5. Fuller CJ, Barr AR, Dieppe PA, et al. Variation of an epitope of keratan sulfate and total glycosaminoglycans in normal equine joints. *Equine Vet J* 1996;28:490–493.

6. Rossdale PD, Hopes R, Digby NJ, et al. Epidemiological study of wastage among racehorses in 1982 and 1983. *Vet Rec* 1985;116:66–69.

7. Little CB, Kilbert NJ, Wickstrom S, et al. Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan in vitro. *Biochem J* 1999;344(part 1):61–68.

8. Palmer JL, Bertone AL, McClain H. Assessment of glycosaminoglycan concentration in equine synovial fluid as a marker of joint disease. *Can J Vet Res* 1995;59:205–212.

9. Alwan WH, Carter SD, Bennett D, et al. Cartilage breakdown in equine osteoarthritis: measurement of keratan sulfate by an ELISA system. *Res Vet Sci* 1990;49:56–60.

10. Alwan WH, Carter SD, Bennett D, et al. Glycosaminoglycans in horses with osteoarthritis. *Equine Vet J* 1991;23:44–47.

11. Tulamo RM, Houttu J, Tupamaki A, et al. Hyaluronate and large molecular weight proteoglycans in synovial fluid from horses with various arthritides. *Am J Vet Res* 1996;57:932–937.

12. Okumura M, Fujinaga T, Urakawa E, et al. Evaluation of the catabolic activity of cartilage by measurement of serum keratan sulfate concentration in foals. *Am J Vet Res* 1997;58:925–929.

13. Okumura M, Tagami M, Fujinaga T. Measurement of serum and synovial fluid keratan sulfate and antibody to collagen type II in equine osteoarthritis. *Zentralbl Veterinarmed A* 1998;45:513–516.

14. Vilim V, Fosang AJ. Characterization of proteoglycans isolated from associative extracts of human articular cartilage. *Biochem J* 1993;293:165–172.

15. Myers SL, Brandt KD, Albrecht ME. Synovial fluid glycosaminoglycan concentration does not correlate with severity of chondropathy or predict progression of osteoarthritis in a canine cruciate deficiency model. *J Rheumatol* 2000;27:753–763.

16. Dey P, Saphos CA, McDonnell J, et al. Studies on the quantification of proteoglycans by the dimethylmethylene blue dye-binding method. *Connect Tissue Res* 1992;28:317–324.

17. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res* 1982;9:247–248.

18. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986;883:173–177.

19. Templeton DM. The basis and applicability of the dimethylmethylene blue binding assay for sulfated glycosaminoglycans. *Connect Tissue Res* 1988;17:23–32.

20. Müller G, Hanschke M. Quantitative and qualitative analyses of proteoglycans in cartilage extracts by precipitation with 1,9-dimethylmethylene blue. *Connect Tissue Res* 1996;33:243–248.

21. Goldberg RL, Kolibas LM. An improved method for determining proteoglycans synthesized by chondrocytes in culture. *Connect Tissue Res* 1990;24:265–275.

22. Møller HJ, Heinegard D, Poulsen JH. Combined Alcian blue and silver staining of subnanogram quantities of proteoglycans and glycosaminoglycans in sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem* 1993;209:169–175.

23. Chandrasekhar S, Esterman MA, Hoffman HA. Microdetermination of proteoglycans and glycosaminoglycans in the presence of guanidine hydrochloride. *Anal Biochem* 1987;161:103–108.

24. Björnsson S. Quantitation of proteoglycans as glycosaminoglycans in biological fluids using an Alcian blue dot blot analysis. *Anal Biochem* 1998;256:229–237.

25. Gold EW. A simple spectrophotometric method for determining proteoglycan concentrations. *Anal Biochem* 1979; 99:183–18.

26. Stone J, Akhtar N, Botchway S, et al. Interaction of 1,9-dimethylmethylene blue with glycosaminoglycans. *Ann Clin Biochem* 1994; 31:147–152.

27. Ayad S. Extracellular matrices. In: Ayad S, ed. *The extracellular matrix factsbook*. Toronto: Academic Press Inc, 1994;1–7.

28. Wight TN, Heinegard DK, Hascall VC. Proteoglycans: structure and function. In: Hay ED, ed. *Cell biology of extracellular matrix*. 2nd ed. New York: Plenum Press, 1991;45–78.

29. Todhunter RJ. Anatomy and physiology of synovial joints. In: McIlwraith CW, Trotter GW, eds. *Joint disease in the horse*. Philadelphia: WB Saunders Co, 1996;1–28.

30. Webb EC, ed. *Enzyme nomenclature 1992. Recommendations of Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on nomenclature and classification of enzymes*. Toronto: Academic Press Inc, 1992;339, 351.