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Examination of synovial fluid and serum following intravenous injections of hyaluronan for the treatment of osteoarthritis in dogs

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Summary
A randomized, blinded, prospective clinical trial was performed to determine the effects of intravenous (IV) administration of hyaluronan sodium (HA) on serum glycosaminoglycans (GAG) concentrations, synovial fluid (SF) hyaluronan concentrations and viscosity in dogs treated for unilateral rupture of the cranial cruciate ligament. Twenty-two dogs undergoing tibial plateau leveling osteotomy were used in this study. Synovial fluid from both stifles and serum were collected prior to surgery and at 2, 4, and 8 weeks following surgery. Dogs received either 1.0 ml (10 mg) of sodium hyaluronate (treatment group 1; n = 10) or equal volume of 0.9% NaCl (treatment group 2; n = 12), IV immediately, 2 and 4 weeks following surgery. Synovial fluid viscosity was evaluated using a magnetically driven, acoustically tracked, translating-ball rheometer. Synovial fluid HA disaccharide content was measured by fluorophore-assisted carbohydrate electrophoresis. Serum GAG concentrations were measured by alcian blue spectrophotometric assay. Data were analyzed using a Wilcoxon sign rank test ($p<0.05$). Mean ± SD viscosity (cP) was significantly higher ($p=0.011$) in SF obtained from the intact stifle (450 ± 604.1) than injured (54.8 ± 60.8) prior to surgery. Mean ± SD HA concentrations (ug/ml) were significantly higher ($p=0.02$) in synovial fluid obtained from the injured stifles (281.4 ± 145.9) than intact stifles (141.6 ± 132.5). No significant difference was noted within or between treatment groups in SF viscosity, HA concentrations, or serum GAG concentrations at any time following surgery. Stifles with cranial cruciate ligament insufficiency had significant alterations in SF viscosity and HA concentrations.

Keywords
Hyaluronic acid, synovial fluid, osteoarthritis

Vet Comp Orthop Traumatol 2005; 18: 169–74

Introduction

Hyaluronan (HA) is a negatively charged, hydrophilic, non-sulphated glycosaminoglycan, composed of repeating units of D-glucuronic acid and N-acetyl D-glucosamine. High molecular weight HA contributes to synovial fluid viscosity and rheologic properties which are crucial for the proper maintenance of joint homeostasis (3, 16, 25, 38, 47, 48). Hyaluronan lubricates joint surfaces (fluid film and boundary lubrication), facilitates dissipation of loads transmitted across articular surfaces, limits migration of inflammatory cells to articular cartilage, and provides protection for articular pain receptors and synoviocytes (30). Without normal HA in the synovial fluid, these functions are compromised and the joint becomes susceptible to the irreversible degenerative changes associated with osteoarthritis (OA) (3).

Investigators have reported that in both humans and animals with OA, the concentration and molecular weight of HA, as well as the viscoelasticity of synovial fluid is abnormally low (3, 4, 9, 16, 25, 26, 30, 38, 47, 48). It appears that the concentration of hyaluronan is decreased by dilution caused by increased synovial fluid volume. The molecular weight of HA molecules decrease due to fragmentation of HA in the synovial fluid or production of abnormally small HA chains by synoviocytes, or both (3, 4, 9, 16, 25, 30, 38, 47, 48). These alterations in HA molecular weight decrease the viscoelasticity of the synovial fluid and impair normal homeostatic articular functions (3, 4, 9, 16, 25, 30, 38, 47, 48).

It has been shown that humans and animals with OA have elevated serum glycosaminoglycan (GAG) concentrations (17, 43). Elevated serum GAG concentrations reflect both an increased rate of GAG production as well as an increase in GAG breakdown products by the synovium and cartilage, respectively (17, 44). From the joint space GAGs pass through the synovial membrane to the lymphatics and then to the bloodstream (44).

Viscosupplementation for the treatment of OA is based on improving the rheological properities within the joint (30, 38). Intra-articular HA has been widely used in the treatment of OA in animals (12, 17, 26, 30, 38, 43) and humans (2, 11, 20, 21, 31). Several clinical studies in humans have demonstrated relief of joint pain associated with OA following intra-articular injections of HA (34, 49). Information regarding the effects of intra-articular HA on naturally occurring OA in dogs is not available, however, several experimental studies using intra-articular HA in dogs have been reported. The results from these studies have demonstrated decreases in pain, lameness, osteophytosis, synovial hyperaemia and hypertrophy, GAG and cartilage degradation (1, 30, 39). The mechanism by which HA produces beneficial effects remains controversial. Hyaluronan has been reported to prevent IL-1 induced proteoglycan release from cultured chondrocytes (41) and carti-

Received September 16, 2004
Accepted March 25, 2005

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lage explants (28, 33), stimulate chondrocytes to synthesize proteoglycans (22), enhance chondrocyte proliferation (22), and enhance collagen synthesis (24). Hyaluronan has been shown to stimulate synoviocyte explants from joints with OA to synthesize HA *in vitro* (42, 43), inhibit PGE_2 synthesis (50), and attenuate endogenous proteinase activities including those of matrix metalloproteinase (45). It has also been shown to alter leukocyte adherence, proliferation, migration, and phagocytosis (13) and mitigates the cytotoxic effects of reactive oxygen species (36).

A preparation of HA (hyaluronan sodium; Legend® Bayer Corporation, Shawnee Mission, KS)[a] has been developed for intravenous administration to alleviate the difficulties associated with repeated intra-articular injections (19). Only two studies have been performed evaluating the safety and efficacy of intravenous HA and both of these studies were in horses. The first study reported a lack of any adverse local or systemic effects from the use of intravenous HA (19). (Package insert, Legend® Bayer Corporation, Shawnee Mission, KS). The second study that was treated with HA reported a significant improvement in lameness, lower concentrations of total protein and PGE_2, and better synovial membrane histologic scores in horses with osteochondral fragments (23).

The purpose of the study reported herein was to evaluate the effects of intravenous HA administration on synovial fluid viscosity, HA concentrations, and serum GAG concentrations in dogs with naturally occurring OA. Our hypothesis was that treatment of dogs with intravenous HA following tibial plateau leveling osteotomy (TPLO, Slocum Enterprises, Eugene, OR) for the treatment of unilateral cranial cruciate ligament insufficiency would increase synovial fluid viscosity, synovial fluid HA concentrations, and decrease serum GAG concentrations compared to saline-treated controls.

[a] Bayer Healthcare LLC., Animal Health Division, Shawnee Mission, KA, USA

Materials and methods

The population for this prospective clinical study consisted of dogs with unilateral cranial cruciate ligament injury. Variables such as age, breed, or sex, and whether the cruciate ligament injury was partial or complete, acute or chronic were not distinguished amongst. The dogs had to be in good overall health and be large enough to be amenable to a TPLO, stabilized with a 3.5 mm TPLO plate (Slocum Enterprises, Eugene, OR). Exclusion criteria for this study included dogs which had received: 1) slow acting disease modifying OA agents, including nutritional joint supplements, such as glucosamine and chondroitin sulphate within 30 days, 2) intra-articular stifle injection within 90 days, 3) surgery on the affected stifle within 180 days prior to presentation for current problem, 4) administration of systemic corticosteroids or antibiotics within 14 days.

This study was conducted under a protocol approved by the University Animal Care and Use Committee and complied with the standards in 'Guide for the Care and Use of Laboratory Animals', prepared by Institute of Laboratory Animal Resources and published by the U.S. National Institute of Health (NIH Publication no. 86–23, revised 1985).

Following initial screening and obtaining client consent, dogs were admitted for surgery. Pre-anaesthetic complete blood cell counts and biochemistry profiles, lameness examination, and TPLO orthogonal view radiographs of both stifles were obtained. Any animal with palpable or radiographic abnormalities suggestive of possible cranial cruciate ligament insufficiency in the contralateral stifle were excluded from this study. The dogs were randomly assigned into one of two treatment groups. The investigators were 'blinded' to treatment group assignments. All of the dogs received etodolac (15 mg/kg PO q 24h) (Etogesic, Fort Dodge, Fort Dodge, IA) 24 h prior to obtaining baseline blood and synovial fluid samples and TPLO surgery. Any that had been receiving non-steroidal anti-inflammatory drugs apart from etodolac prior to admission to the hospital had their medication changed to etodolac.

On the day of surgery, both stifles were aseptically prepared and pre-treatment synovial fluid samples were obtained from both stifles via arthrocentesis with a 0.9 mm needle. Until the time of analysis, synovial fluid specimens were placed in micro centrifuge tubes, and stored at –70°C A 10 ml venous blood sample was collected from each dog and placed in a vacutainer tube and immediately placed on ice. Within 30 minutes, the serum samples were centrifuged for 15 minutes at 3000 x g at 10°C. Serum was removed, divided into two aliquots (1.5 ml each) and frozen in polypropylene tubes at –70°C. Following TPLO, at the time of skin closure dogs in treatment group 1 (n=10) received 1.0 ml (10 mg) of HA via the cephalic vein. The dogs in treatment group 2 (n=12) received 1.0 ml 0.9% NaCl, and were discharged from the hospital two to five days after surgery and continued to receive etodolac for one month. Those dogs did not receive any corticosteroids nor slow acting disease-modifying osteoarthritic agents for the duration of the study.

Each dog was returned to the hospital two weeks after surgery for further sample collections and treatment. They were sedated with acepromazine (0.01 mg/kg IV) and hydromorphone (0.1 mg/kg IV). A venous blood sample and synovial fluid samples were obtained from both stifles and these samples were processed as previously described. Following sample collection, the dogs received a second intravenous injection of either 1.0 ml (10 mg) HA (treatment group 1) or 1.0 ml 0.9% NaCl (treatment group 2). Each dog was again returned four weeks following surgery for a third synovial fluid and serum collection, intravenous treatments (HA – treatment group 1, NaCl – treatment group 2), and stifle radiographs were obtained. Final synovial fluid, serum samples, and stifle radiographs were obtained at eight weeks following surgery.

Synovial fluid viscosity

Synovial fluid viscosity was determined using a magneto-acoustic ball microrheometer as previously described, using a small cylindrical magnet with a diameter of 1.0 mm (46). The terminal velocity of the cylin-

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der is inversely proportional to the viscosity. The tube was surrounded by a water bath for accurate temperature controls. The small magnetic cylinder was elevated by a small electromagnet coupled with a micromanipulator, and the cylinder's position was tracked by ultrasonic pulse-echo method. The ultrasonic pulse-echo transducer transmits a single sound pulse and acts as a receiver when the sound wave is reflected off the magnet. The cylinder displacements were measured using a system consisting of a time-to-voltage converter, a pulse generator, a differential amplifier, and an oscilloscope (150 Mhz). The microrheometer measured the viscosity of synovial fluid in the very short 10 mm long chamber. Distilled water (viscosity of 1 centi Poise) was used as a standard.

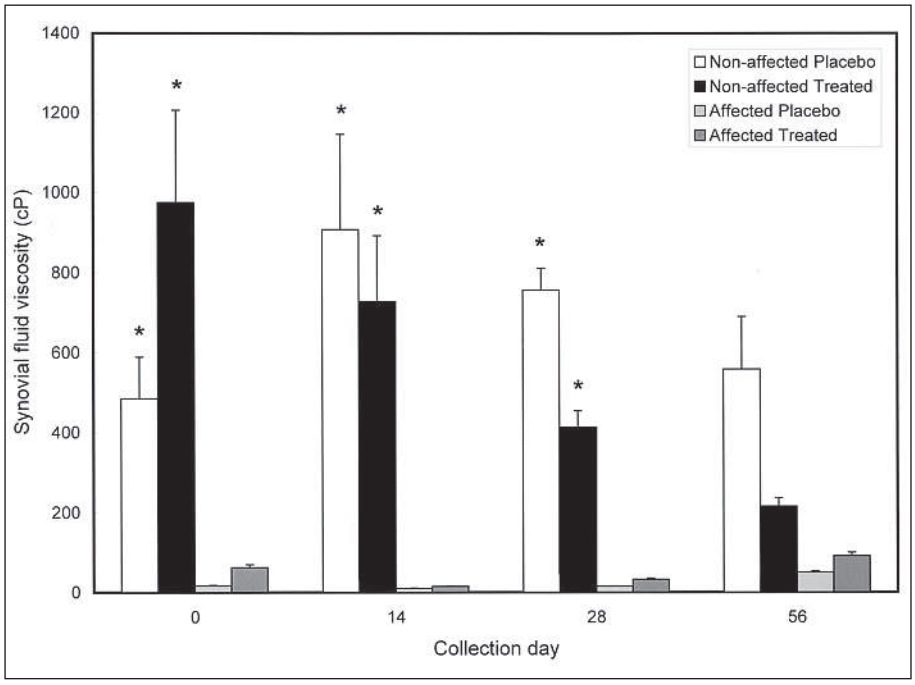

Fig. 1 Mean (+/- SD) synovial fluid viscosity (cP) levels obtained by arthrocentesis at days 0, 14, 28, and 56. Asterisks indicate the days on which a significant difference was noted between samples obtained from injured and intact stifles ($p < 0.05$).

Hyaluronan disaccharide concentrations

The unsaturated disaccharides of hyaluronan (Δ diHA) were quantified in synovial fluid samples using fluorophore-assisted carbohydrate electrophoresis (FACE), as previously described, with the following modifications (7). Synovial fluid (500 µl) was added to an equal volume of 0.1 M Na acetate (pH 7.0) and 800 µg of proteinase K and then digested for 6 h at 60°C. After enzyme inactivation at 100°C for 10 min, the pH was adjusted to 6.0 with glacial acetic acid. Hyaluronidase (Streptomyces hyalurolyticus; 2 turbidity reducing units) was added and the sample digested overnight at 60°C. After heating to 100°C for five min to inactivate the enzyme, the sample was applied to a Sephadex G-50 (Amersham Pharmacia Biotech, Amersham Biosciences Corporation, Piscataway, NJ) column pre-conditioned with 1 ml of bovine serum albumin (10 mg/ml). Glycosaminoglycans were eluted in water (sample loaded (Fraction 1), followed by ten 500 µl fractions of water). Fractions 7 to 11 (HA fractions) were collected and pooled. The HA eluent was dried in a centrifuge concentrator and then resuspended in 100 µl of 100 mM ammonium acetate (pH 7.3). After adding chondroitinase ACII (50 mU), the samples were incubated at 37°C for 3 to 4 h. For HA analysis, 0.5 % of the digest was dried in preparation for labeling with 2-aminoacridone (AMAC) and sodium cyanoborohydride, as previously described (34). Internal standards (galactose 6S at 60, 120, 240, and 480 pmol) were prepared and labeled with AMAC. After labeling was complete, 40 µl of 25% glycerol were added to samples and standards. A portion (2 ul standard, 4 ul sample) was loaded on a polyacrylamide gel (MONO composition gel; Glyko) for FACE separation. Electrophoresis was performed at 500 V for 75 min at 4°C. Gels were imaged and quantitated using the Kodak 1-D System (KDS, Kodak, Rochester, NY). Identification of bands was confirmed with HA standard.

Serum GAG concentrations

Glycosaminoglycan concentrations in serum samples were determined with an Alcian blue spectrophotometric assay as previously described (6). 100 µl aliquots of serum were centrifuged in 1 ml of 0.1% Alcian blue solution for three minutes at 3000 x g. Then 500 µl of the supernatant were added to 3 ml PBS, vortexed and the absorbance read at 620 nm. Concentrations of GAG were determined from a standard curve generated using a commercial hyaluronate sodium standard (Legend, Bayer Corporation, Shawnee Mission, KS).

Statistical analysis

The differences in synovial fluid viscosity, synovial fluid HA concentrations, and serum GAG concentrations were compared between treatment groups and between the injured versus contra-lateral intact stifle before and 14, 28, and 56 days following surgery using a Wilcoxon sign rank test. Significance was based at $p \leq 0.05$.

Results

Synovial fluid viscosity

The pre-operative (day 0) mean synovial fluid viscosity was significantly higher ($p=0.011$) in synovial fluid specimens ob-

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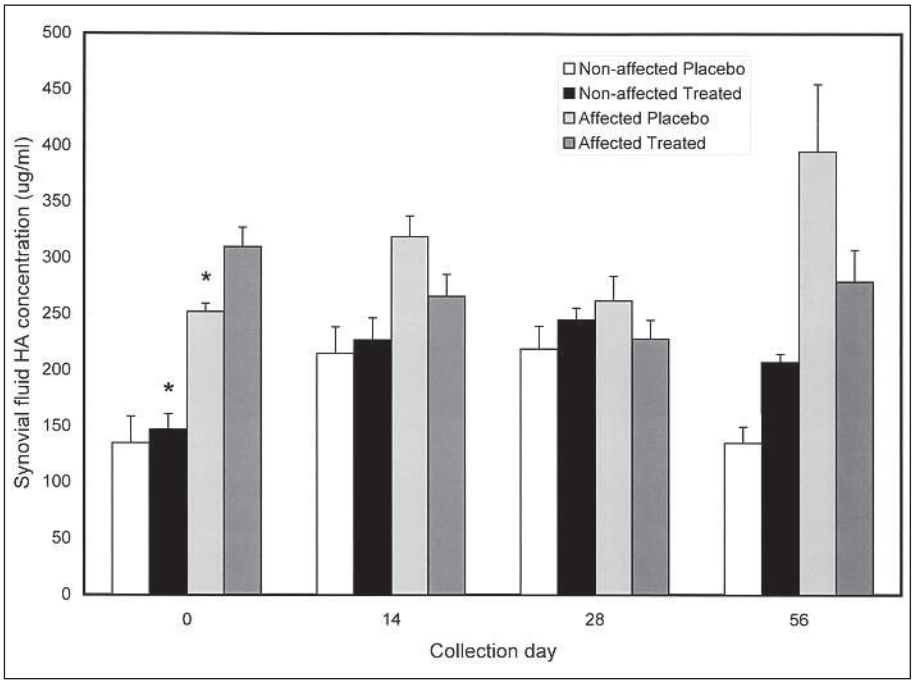

Fig. 2 Mean (+/- SD) synovial fluid HA levels (ug/ml) obtained by arthrocentesis at days 0, 14, 28, and 56. Asterisks indicate the days on which a significant difference was noted between samples obtained from injured and intact stifles ($p<0.05$).

tained from the intact contralateral stifles (450.09±604.10 cP) when compared to injured stifles (54.82±60.81 cP) (Fig. 1). The synovial fluid viscosity remained significantly higher in intact contralateral stifles than the injured stifles at days 14 and 28 following surgery (Fig. 3), and it did not change significantly over time within the treatment groups (intact contralateral or injured stifles). A significant difference was not found between HA treated and placebo treated synovial fluid viscosity in either injured or intact stifles at any point in time.

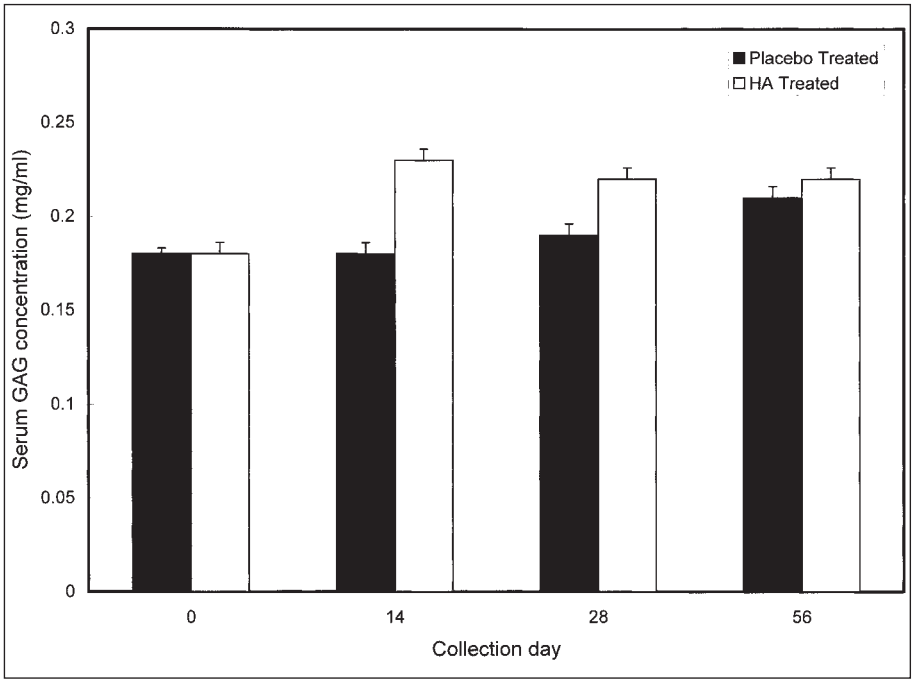

Fig. 3 Mean (+/-) serum HA levels (ug/ul) at days 0, 14, 28, and 56.

Synovial fluid HA concentrations

Pre-operative (day 0) mean HA concentrations were significantly higher ($p=0.02$) in synovial fluid specimens obtained from injured stifles (281.45±145.96 µg/ml) compared to the intact contra-lateral stifles (141.14±132.48 µg/ml) (Fig. 2). Synovial fluid HA concentrations did not significantly change over time within either treatment groups (intact contralateral or injured stifles). A significant difference in synovial fluid HA concentrations was not noted between treatment groups in either the injured or intact contra-lateral stifles at any time.

Serum GAG concentrations

All of the dogs in the study possessed clinical and radiographic evidence of stifle instability, and therefore control serum GAG concentrations were not available. Pre-operative (day 0) mean serum GAG concentrations were 0.17±0.03 ug/ul and the Serum GAG concentrations did not change significantly over time within or between treatment groups (Fig. 3).

Discussion

This is the first report that particularly quantitates synovial fluid viscosity values in dogs. Previous quantitative reports of synovial fluid viscosity values in humans have utilized assays such as the mucin clot test (37), thumb test (18), and the white blood cell diluting pipette (15). In this study, the synovial viscosity was determined using the magneto-acoustic ball microrheometer, which had been developed by one of the co-investigators (46). This novel instrument requires a much smaller sample volume (approx. 20 µl) than traditional viscometers, and opaque suspensions can also be studied.

In our study, we found a significant decrease in the synovial fluid viscosity in in-

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jured stifles, when compared to intact contralateral stifles. The high variability of synovial fluid viscosity noted in the contralateral intact stifles is consistent with values reported in human patients, in which normal synovial fluid viscosity has been shown to range from 6 to 1200 cP (27). It is questionable that human patients with synovial fluid viscosity values of 6 cP are normal because the mucin clot test used to determine viscoelasticity deteriorates at 20 to 30 cP (27). The lowest viscosity value in samples obtained from the contra-lateral intact stifle in the dogs in our study was 38 cP.

In a previous study, intra-articular HA caused a significant increase in synovial fluid viscosity in humans with OA of the knee. In that study, the rheological properities of synovial fluid were examined following five weekly intra-articular HA or saline treatments (32). Median values for the shear storage modulus (a measure of viscosity) increased in patients receiving HA, but were unaltered in the saline control group. Compared to our 56 day evaluation, patients in that report were evaluated for six months (32).

The normal molecular weight of synovial fluid HA has been reported to be approximately 7 x 10^6 Da in humans, 3 x 10^6 Da in horses, and 7–9 x 10^5 Da in dogs (29, 44, 48). In dogs with OA, the concentration and molecular weight of HA in synovial fluid is markedly diminished (30). The concentration of HA may be decreased by dilution secondary to exudation (30). Due to fragmentation of HA in the synovial fluid and production of abnormally low molecular weight HA by synoviocytes, the size of HA molecules decreases. These changes diminish the viscoelasticity of the synovial fluid and impair the joint haemostatic functions of HA. Most studies evaluating HA concentrations in synovial fluid utilize assays such as high-performance liquid chromatography (5, 47) or enzyme-linked immunosorbent-inhibition assay (25). These assays are designed to detect intact HA molecules in the synovial fluid and therefore reveal a decreased HA concentration in joints with OA. The FACE assay utilized in this study measures HA disaccharide fragments. At day 0, total HA concentration in injured stifles (281.45±145.96 µg/ml) was significantly different from the contralateral intact stifles (141.64±132.48 µg/ml).

Due to the fact that all of the dogs in our study had clinical and radiographic evidence of stifle instability, we were unable to compare serum HA concentrations in dogs with OA and normal dogs. Pre-operative serum GAG concentrations in our population of dogs (0.17 +/- 0.03 µg/ul ; range 0.07-0.38 µg/ml) was similar to that of previously reported in dogs with carpal synovitis (0.32 +/- 0.02 µg/ul ; range 0.18-0.29 µg/ml) using the Alcian blue assay (8). Due to different OA models, although the results from these two studies cannot be compared directly, they do illustrate the ranges that may be expected in dogs using the Alcian blue assay. A significant difference in serum GAG concentrations was not noted between, or within, treatment groups following intravenous HA administration. The timing of serum sample collection may have affected our results. Humans have a diurnal variation in serum GAG concentrations. In normal individuals and patients with rheumatoid arthritis, the highest values occur in the morning, one hour after arising and performing routine morning activities (40). In the present study, the dogs returned for follow-up examinations during different time periods throughout the day and a set collection time was not established.

Following the TPLO procedure, dogs are commonly 'toe-touching' to 'non-weight-bearing' lame for variable periods of time (10). During the post-operative convalescence period, the contra-lateral intact stifle is subject to increased loads. An increased loading of the stifle has been shown to result in formation of osteophytosis, more severe histological evidence of cartilage damage, and reduction in the proteoglycan concentration in cartilage (14).

Our study was performed with an HA preparation (Legend, Bayer Corporation, Shawnee Mission, KS) that has not been approved in the United States for intravenous use in dogs. Intravenous administration of HA has been evaluated in horses. Local or systemic side effects were not reported (23) (Legend package insert, Bayer Corporation.) Unpublished data from the manufacturer of the HA product used in our study did not reveal any adverse events from the IV administration to dogs. Currently, there is not an established dosing regimen for intravenous HA in dogs. The recommended dose for intravenous HA (legend) in horses is one 40 mg (10 mg/ml) injection weekly for three weeks. The recommended dose for intra-articular HA treatment in horses (20 mg x 3 treatments) (legend insert). Extrapolating from the dosing schedule in horses, the intravenous dosing schedule used in this study was similar to the intra-articular dose used in dogs (one 10 mg (10 mg/ml) injection weekly for three treatments). However, in order to fit the recheck examination schedule, we administered a dose every two weeks rather than weekly. Other studies evaluating the effects of intra-articular HA in dogs have used higher doses (10–20 mg) and longer treatment periods (7 to 16 weekly injections) (23, 26).

In our study, a series of three intravenous HA injections (every other week) did not result in any significant change when compared to placebo treatment in synovial fluid viscosity, HA concentration, or serum GAG concentrations. The reasons that may account for the lack of any significant change may include timing of blood collection, inappropriate dosing schedule, small population size and the short duration of the study. Although the viscosity and HA concentrations did not differ between HA-treated and placebo-treated dogs, the significant differences between normal and affected SF viscosity and HA concentrations found within this study, and the assays utilized, may be useful for future etiological and therapeutic investigations.

Acknowledgements
We wish to thank Dr. Clark Billinghurst, April Childress, Bryan Conrad, Sheri Holloway, James van Gilder, and Debby Sunstrom for assistance with this study.

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