IN VIVO KINETIC STUDY ON UPTAKE AND DISTRIBUTION OF INTRAMUSCULAR TRITIUM-LABELED POLYSULFATED GLYCOSAMINOLGYCAN IN EQUINE BODY FLUID COMPARTMENTS AND ARTICULAR CARTILAGE IN AN OSTEOCHONDRAL DEFECT MODEL

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SUMMARY

The uptake and distribution of intramuscularly (IM) administered tritium-labeled polysulfated glycosaminoglycan (3H-PSGAG) in serum, synovial fluid, and articular cartilage of eight horses was quantitated, and hyaluronic acid (HA) concentration of the middle carpal joint was evaluated in a pharmacokinetic study. A full-thickness articular cartilage defect, created on the distal articular surface of the left radial carpal bone of each horse served as an osteochondral defect model. 3H-PSGAG (500 mg) was injected IM, between 14 and 35 days after creation of the defects. Scintillation analysis of serum and synovial fluid, collected from both middle carpal joints at specific predetermined times up to 96 hours post-injection, revealed mean 3H-PSGAG concentrations peaked at 2 hours post-injection. 3H-PSGAG was detected in cartilage and subchondral bone 96 hours post-injection in samples from all eight horses. There were no statistically significant differences in 3H-PSGAG concentration of synovial fluid or cartilage between cartilage defect and control (right middle carpal) joints.

HA assay of synovial fluid revealed concentrations significantly increased at 24, 48, and 96 hours post-injection in both joints. The concentration nearly doubled 48 hours post-injection. However, no statistically significant differences were found between synovial concentrations of HA in cartilage defect and control joints.

3H-PSGAG administered IM to horses, was distributed in the blood, synovial fluid, and articular cartilage. HA concentrations in synovial fluid increased after IM administration of polysulfated glycosaminoglycan.

INTRODUCTION

Polysulfated glycosaminoglycan (PSGAG) is used in the treatment of ostearthritis in horses. PSGAG consists of a polymeric chain of alternating units of hexuronic acid and hexosamine with a molecular weight of approximately

*Adequan, Luitpold Pharmaceuticals, Inc., Shirley, NY.
10,000 daltons.\textsuperscript{1,2} It is chemically related to heparin and the glycosaminoglycans (GAG's) in articular cartilage.\textsuperscript{1} PSGAG\textsuperscript{2} is extracted and purified from bovine tracheal tissue and approved for both intra-articular (IA) or intramuscular (IM) administration in horses.\textsuperscript{1}

PSGAG is considered to have a chondroprotective effect, because of its ability to inhibit proteoglycan and collagen degrading enzymes. In vivo and in vitro studies indicate PSGAG inhibits lysosomal enzymes, neutral protease, stromelysin (a neutral metalloproteinase), and neutral serine proteinase (elastase).\textsuperscript{3-6} Other reports indicate the presence of supernoxide radical and interleukin-1 inhibitory properties.\textsuperscript{7,8} Less articular cartilage fibrillation, erosion, chondrocyte death, and greater safranin-O staining for GAG's occurred in horses with chemically induced cartilage damage when PSGAG was administered IA, compared to control joints not injected with PSGAG.\textsuperscript{9} PSGAG is described as having anti-inflammatory and possibly analgesic benefits attributable to decreased prostaglandin E\textsubscript{2} release as an indirect result of inhibition of lysosomal enzyme liberation.\textsuperscript{10,\textsuperscript{11}} A decrease in polymorphonuclear leukocytes and an increase in lymphocytes in synovial fluid have been observed after IA injection of PSGAG in humans.\textsuperscript{12,13} In vitro studies describe PSGAG as having a stimulatory effect on collagen, proteoglycan, and hyaluronic acid (HA) synthesis, as well.\textsuperscript{14-16}

Originally, PSGAG was designed as an IA medication for horses. Transient joint inflammation and associated lameness has been occasionally observed after IA administration. Recent reports have indicated that IA use of PSGAG increased the incidence of sepsis in equine carpal joints injected with subinfective doses of \textit{Staphylococcus aureus}.\textsuperscript{17,18} Subsequently, PSGAG was found to be effective when administered IM.\textsuperscript{19} IA and IM use in human and equine clinical studies have resulted in no significant differences in response.\textsuperscript{20,21} Improvement in range of joint motion and decreased joint pain have been reported in humans and animals after IM administration of PSGAG.\textsuperscript{16,19,22,23} There have been anecdotal reports of improved clinical response in horses with non-infectious degenerative joint disease (DJD) following oral or IM administration of various dosages of PSGAG.\textsuperscript{19}

Numerous studies have reported the ability of PSGAG achieving therapeutic concentrations in synovial fluid and articular cartilage in humans and laboratory animals after IM administration.\textsuperscript{16,22-32} However, the uptake and distribution of tritium-labeled PSGAG (\textsuperscript{3}H-PSGAG) administered IM in horses has not been reported previously. The purposes of this study were to determine the systemic uptake and distribution of \textsuperscript{3}H-PSGAG into equine body fluid compartments and articular cartilage after IM administration and to evaluate the effect of IM PSGAG on the HA concentration in synovial fluid of normal joints and joints with osteochondral defects.

**MATERIALS AND METHODS**

**Horses**

Eight mature horses (5 mares, 3 geldings, ages 2 to 14 years) with clinically and radiographically normal carpi were used in the study.

**Creation of Osteochondral Defect Model**

The horses were sedated with xylazine\textsuperscript{b} (0.22 mg/kg, IV), and anesthesia induced with 5% guaifenesin\textsuperscript{c} (30 to 35 gm) in combination with thiopental sodium\textsuperscript{d} (6.6 mg/kg, IV). The horses were intubated, maintained on halothane\textsuperscript{e}-oxygen anesthesia, and placed in dorsal recumbency. The left carpus was aseptically prepared, draped and suspended for arthroscopic surgery. A full thickness cartilage defect (approximately 5 mm x 10 mm) was created using arthroscopic technique\textsuperscript{29} with a 4 mm motorized arthroturr,\textsuperscript{1} on the distal dorsomedial articular surface of the left radial carpal bone of each horse. No osteochondral defect was made on the right radial carpal bone, which served as the non-osteochondral defect control (referred to as control throughout the text). Records of articular cartilage surfaces in the right and left middle carpal joints, both prior to and after creation of defect, were compiled on videotape\textsuperscript{a} and computer video discs\textsuperscript{a} via a 4 mm, 25° arthroscope\textsuperscript{c} and camera.\textsuperscript{1}

**Tritium-labeling of the PSGAG**

PSGAG was tritiated unspecifically in aqueous solution with tritium gas using palladium oxide as catalyst. The \textsuperscript{3}H-PSGAG was purified by chromatography on Bio Gel P30\textsuperscript{k} with 2 N sodium chloride as eluent. The product was subsequently desalted by chromatography on Bio Gel P2\textsuperscript{k} with water.

The molecular weight (MW) of the \textsuperscript{3}H-PSGAG was determined chromatographically with 2 N sodium chloride of a sample on Bio Gel P30 column, comparing with the elution pattern of MW standard substances. Dimethylene blue chemical determination of position of the peak yielded a MW of 5300 daltons, while radiometric determination yielded 4300 daltons. Electrophoresis of PSGAG and \textsuperscript{3}H-PSGAG on cellulose acetate foils using 35 mM sodium barbital as buffer showed homogenous spots with the same mobility.\textsuperscript{30}

\textsuperscript{a}Rompun, Bayvet Division, Cutter Laboratories, Shawnee Mission, KS.
\textsuperscript{b}Geocolate, Summit Hill Laboratories, Navesink, NJ.
\textsuperscript{c}Bio-Tal, Bio-ceutin Laboratories, St. Joseph, MO.
\textsuperscript{d}Halothane, Fort Dodge Laboratories, Fort Dodge, IA.
\textsuperscript{e}Wolf PAC-3000, Richard Wolf Medical Instruments Corp., Rosemont, IL.
\textsuperscript{f}Minolta 0.5° SVHS System, Minolta Corp., Denver, CO.
\textsuperscript{g}Videc Floppy System, Hitachi, Elk Grove Village, IL.
\textsuperscript{h}Panorview +, Richard Wolf Medical Instruments Corp.
\textsuperscript{i}Arthroscopic Video Camera, Dynoics Video Division, Oklahoma City, OK.
\textsuperscript{j}Bio Rad Co., Rockville Centre, NY.
unlabeled PSGAG (4436.5 mg), in physiological saline to a total volume of 9.0 ml. The solution was subsequently sterilized by allowing it to pass through a 0.25 μm Millex GV filter to yield 8 ampoules of 1.0 ml each, containing 500.0 mg \(^3\)H-PSGAG with a specific radioactivity of 1.739 mCi (64.3 MBq) and 3.806 x 10\(^8\) disintegrations per minute (dpm).

**Drug Injection and Sample Collection**

The horses were paired and an identical sample collection protocol was followed for each of the 4 pairs (Table 1). Each horse was injected with a single 500 mg dose of \(^3\)H-PSGAG into the serratus ventralis cervicis muscle (left lateral cervical region), 4 cm cranial to the cranial border of the scapula. The 4 pairs were injected at different times after creation of cartilage defects: pair 4 was injected at 14 days, pair 3 at 21 days, pair 2 at 28 days, and pair 1 at 35 days post defect creation. This detailed documentation of the injection times was made for each pair to allow observation for any possible influence age of cartilage lesion may have on \(^3\)H-PSGAG concentration in the synovial fluid or articular cartilage.

Urine, blood, and synovial fluid (left and right middle carpal joints) were collected into plain glass collection tubes prior to injection (time = 0 hour), and at 2, 4, 8, 12, 24, 48, and 96 hours post-injection (Table 1). Synovial fluid (2 ml) was obtained with the animal standing, using the lateral approach.\(^3\) The blood samples were allowed to clot at room temperature, and serum was separated by centrifugation. Serum and urine were stored at 4°C and synovial fluid samples at -20°C until analyzed.

The horses were monitored daily and handwalked to assess for evidence of joint pain related to the sample collections. Phenytoin (4.4 mg/kg PO, q 24 h) was administered if lameness was noted.

The horses were sacrificed 96 hours post-injection. The middle carpal joints were disarticulated and the entire distal articular cartilage surface, with 3 to 4 mm of attached subchondral bone, of the left and right radial carpal bones were collected. These samples were sectioned sagittally through the 2nd carpal facet of the articular surface and fixed in 70% ethanol for at least 24 hours.

**Scintillation Counting of Tritium in Body Fluids**

A 200 μl sample of urine, serum, or synovial fluid was added to 8 ml of scintillation fluid\(^6\) and counted in a liquid scintillation spectrometer.\(^7\) \(^3\)H-m-hexadecane\(^8\) was used as the counting standard in the calculations of the internal counting efficiency for each fluid type. All data were corrected for background and counting efficiency and depicted as dpm of radioactivity per 200 μl sample. The levels of radioactivity were converted to concentration of \(^3\)H-PSGAG per sample and expressed as μg/ml, using the following formula:

\[
\frac{(500 \text{ mg} \ \text{PSGAG/3.806x10}^8 \text{ dpm}) \times (1000 \mu\text{g/mg}) \times (R_{BG}/200 \mu\text{l}) \times (1000 \mu\text{l/ml})}{R_{BG} \text{ dpm of radioactivity in body fluid sample}} = \mu\text{g/ml}
\]

**Scintillation Counting of Tritium in the Cartilage and Subchondral Bone Harvested at Necropsy**

The cartilage from the distal articular surface of the radial carpal bones harvested at necropsy (96 hours post-injection), was separated from the subchondral bone. Three samples of both the cartilage and subchondral bone from each radial carpal bone were then taken and each sample weighed in a gelatin capsule (lock caps size 3). Three capsules each with 100 mg samples were prepared. Each capsule was enveloped in a 2 cm x 2 cm slow match with an overlapping end of 2 cm.

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1. Scintiverse II, Fisher, Plano, TX.
2. Tracer Analytic Delta 300, Elk Grove Village, IL.
3. Amersham TRR 6, Arlington Heights, IL.
x 0.5 cm, cut out of residue and lignin free filter paper. The capsule was subsequently positioned into a platinum basket fixed to a 29 mm glass stopper of a 500 ml Erlenmeyer flask. The stopper was greased with non-burning silicone. After filling the flask with oxygen, the slow match was lit and the stopper was fixed to the flask by a spring clamp. After the completed combustion, the closed flask was stored for 20 minutes at -20°C. Scintillation cocktail (18 ml) was added, and the closed flask was shaken vigorously for 2 minutes. After standing and warming to room temperature, the flask was shaken again for 10 seconds. A 15 ml aliquot was transferred to a scintillation counting vial and stored for a minimum of 16 hours at room temperature to allow chemiluminescence to decay. After counting for 5 minutes, the measurement was repeated after adding an internal standard of tritium-water. Background levels of radioactivity were determined by oxidation and counting of non-radioactive control samples. Data was expressed as µg/gm using the same formula as for the body fluid samples with the exceptions being R representing the dpm of radioactivity per mg of cartilage (Rc) or subchondral bone (Rb) and dry measures (mg) used instead of fluid.

Hyaluronic Acid Assay

Synovial fluid was assayed for HA by a modification of a previously described dye-binding method. A standard curve was prepared as follows: from a stock solution of 2 mg/ml HA in 0.9% saline, dilutions were made to give 2, 10, 20, and 30 µg/tube. One ml of Alcian blue reagent (20 mg/ml in 45 mM sodium acetate, pH 5.8, 50 mM MgCl2) was added, followed by incubation at room temperature for 5 to 30 minutes. Absorbance of transmitted light at the wavelength of 620 nm was measured after dilution with 3 ml of 0.015 M phosphate buffer (pH 7.5), containing 0.15 M NaCl and 0.003 M KCl. Triplicate standards were analyzed and the correlation coefficient for each curve (total of 4 runs) was calculated to greater than 0.999. Internal reference standards of 1 mg/ml HA, 10 mg/ml bovine serum albumin were run in duplicate with each assay and varied no more than 6% from the standard value. Synovial fluid samples were analyzed in duplicate. If duplicates varied by more than 6% from their average, the sample was reanalyzed.

Statistical Analysis

Concentrations of 3H-PSGAG in the urine, serum, and in the synovial fluid from both the cartilage defect (left) joints and control (right) joints were described by a one-compartment open pharmacokinetic model for IM injections: \[ Y = B_0 k_\alpha [\exp (k_\alpha t) - \exp (-k_\alpha t)]/(k_\alpha - k_E) \], where \( Y \) is the given response, \( t \) the time (hours) following injection, \( k_\alpha \) the absorption rate constant, \( k_E \) the elimination rate constant, and \( B_0 \) a scale parameter. The model parameters were estimated by non-linear, least-squares, regression analysis for each horse and for the mean over all the horses using the NONLIN module of SYSTAT. However, the area under the concentration-time curve (AUC) in each of these cases was estimated by the non-parametric trapezoidal method. For each of the above responses, on each horse, the observed maximum concentration (CMAX) and the sampling time at which the maximum occurred (TMAX) were recorded as variables characterizing the response. The information provided by each set of 7 concentrations (observed at 2 through 96 hours) was transformed into estimates of 6 parameters: \( B_0, k_\alpha, k_E, \text{AUC, CMAX and TMAX} \).

Hyaluronic acid in the synovial fluid was not modeled by the above equation. The AUC was estimated and the maximum concentration and time of maximum concentration were recorded for control and cartilage-defect joints on each horse and the mean over all horses. For any of the measures mentioned, the AUC is taken to be proportional to the average ‘exposure’ of the joint to the substance being measured. The hyaluronic acid AUC was plotted versus each of the six parameter estimates (described above) for urine and for the synovial fluid in each joint to see if any correlations between these parameters and ‘average’ hyaluronic acid levels might be suggested.

Synovial fluid concentrations of 3H-PSGAG and hyaluronic acid over hours 0 through 96 were analyzed by repeated measures of analysis of variance. Tests were made for differences between control and cartilage defect joints at each sampling time. For hyaluronic acid concentrations, the significance of the change from baseline (0 hour sample) was assessed at each sampling time.

A separate repeated measures analysis of variance was done for the 96 hour levels of 3H-PSGAG in subchondral bone and cartilage samples. Differences between control and cartilage-defect joints were tested.

RESULTS

Effects of the Study on the Animals

None of the horses exhibited any adverse reaction systemically or locally as a result of the 3H-PSGAG injection. Mild lameness was noted after performing repeated arthrocenteses.

Scintillation of the Body Fluids, Cartilage Samples, and Subchondral Bone

Scintillation analysis revealed 3H-PSGAG in the urine, in the serum, and in the synovial fluid of both the cartilage defect and control joints. Of the times urine was collected, the mean concentration of 3H-PSGAG was highest 2 hours post-injection. By 24 hours, most of the drug elimination via the urine occurred (Fig. 1).

Mean concentrations of 3H-PSGAG in the serum and synovial fluid peaked 2 hours post drug injection, then rapidly

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8SYSTAT Inc., 1800 Sherman Blvd., Evanston, IL.
declined over 24 hours post-injection (Fig. 2). The mean concentrations thereafter remained fairly constant for the remainder of the 96 hour test period. The peak mean concentration in the serum was 1.958 ± 0.197 μg/ml. The peak mean in the synovial fluid of the cartilage defect joints was 0.331 ± 0.049 μg/ml, and 0.248 ± 0.043 μg/ml in the synovial fluid of the control joints. The difference in the peak mean 3H-PSGAG concentrations between cartilage defect and control
joints was not statistically significant. No significant differences in the synovial fluid $^3$H-PSGAG concentration were found between the cartilage defect and control joints at any sample time.

Concentrations of $^3$H-PSGAG were detected in the cartilage of all eight horses 96 hours post drug injection. The concentration in the cartilage defect joints ranged from 0.204 to 0.456 μg/gm with a mean of 0.290 ± 0.029 μg/gm, and ranged from 0.149 to 0.628 μg/gm with a mean of 0.303 ± 0.051 μg/gm in the control joints (Fig. 3). There were no statistically significant differences in the cartilage $^3$H-PSGAG concentration between the cartilage defect and control joints. Detectable levels of $^3$H-PSGAG were also found in the subchondral bone 96 hours post drug injection (Fig. 3). The mean concentrations in subchondral bone of the cartilage defect and control joints were 0.161 ± 0.018 and 0.144 ± 0.019 μg/gm, respectively. There were no significant differences in subchondral bone $^3$H-PSGAG concentration between the cartilage defect and control joints.

**Hyaluronic Acid Assay**

The HA concentration in the cartilage defect and control joints showed an increase 24 hours after administration of $^3$H-PSGAG (Fig. 4). Hyaluronic acid levels in both the cartilage defect and control joints were significantly (P=0.001 and 0.041) different from the pre-injection values (0-hour) at 24, 48, and 96 hours post injection. The mean pre-injection HA concentration for the cartilage defect joints was 0.438 ± 0.062 mg/ml and 0.476 ± 0.087 mg/ml for the control joints. The mean HA concentrations of the cartilage defect and control joints respectively, 24 hours post-injection were 0.653 ± 0.027 and 0.660 ± 0.072 mg/ml; at 48 hours post-injection were 0.933 ± 0.097 and 0.921 ± 0.113 mg/ml; and at 96 hours post-injection were 0.775 ± 0.111 and 0.865 ± 0.118 mg/ml. However, there were no significant differences in the HA concentrations between cartilage defect and control joints at any sample time.

**DISCUSSION**

Scintillation analysis was used to measure the amount of tritium activity in the samples. It was presumed that the tritium was still attached to the PSGAG in this study; work conducted by others, suggests the plausibility of this presumption.37

Concentrations of PSGAG sufficient to inhibit cartilage degrading enzymes in equine DJD have not been established. Concentrations of PSGAG necessary to inhibit certain degrading enzymes (ie, neutral protease, β-glucuronidase, elastase) and to stimulate proteoglycan biosynthesis have been established in vitro;4,8,38,39 inhibition of elastase and neutral protease has been noted with PSGAG concentrations as low as 0.1 μg/ml,48 and β-glucuronidase may be inhibited with concentrations of approximately 1 μg/ml.39

In vitro studies have shown the stimulatory effects of PSGAG on matrix synthesis are dose-dependent.17,40 PSGAG concentrations of 0.3 to 1 μg/ml are adequate to stimulate proteoglycan synthesis in diseased articular cartilage.38 Osteoarthritic cartilage appeared more sensitive than normal cartilage to stimulation of proteoglycan synthesis by exogenous PSGAG in in vitro studies.15,17 A recent report however showed that PSGAG concentrations of 50 and 200 μg/ml exerted no significant effect on proteoglycan synthesis in equine articular explants.41

$^3$H-PSGAG concentrations in the articular cartilage samples obtained in this study were of the level reported by others (0.1 μg/ml) to inhibit certain cartilage degrading enzymes. The $^3$H-PSGAG concentrations in some cartilage samples was also of the levels reported (0.3 μg/ml) to stimulate proteoglycan synthesis. However, the therapeutic usefulness of PSGAG depends on the progress and severity of cartilage degeneration.

Considerable increase in the HA concentration of the synovial fluid occurred during this experiment. This was also observed during treatment of human osteoarthritis patients with PSGAG administered IM.16 An increase in HA concentration in synovial fluid of cubital joints in lame boars was also observed after administration of PSGAG IM.23 This may have been caused by competitive inhibition by PSGAG of the catabolism of HA, and a stimulation of compensatory biosynthesis of high polymeric HA from UDP-N-acetylglucosamine and UDP-glucuronic acid.5,9 Chondrocytic HA synthesis has been stimulated in vitro by addition of PSGAG to either culture medium16,18,42 or synovial fluid from osteoarthritis patients.43 It has been suggested that PSGAG may link molecules of HA, forming complexes of high MW HA.43

Concentrations of $^3$H-PSGAG in the urine, serum and synovial fluids were adequately described by the proposed pharmacokinetic model. However, no obvious correlations were found between hyaluronic acid levels in the synovial fluid and either the estimated model parameters or any other transformation of the raw $^3$H-PSGAG concentrations in the urine, serum or synovial fluids. Consequently, no mechanism was suggested for a causal relationship between drug concentrations in body fluids and hyaluronic acid concentrations in the synovial fluids. Nevertheless, this study showed that $^3$H-PSGAG administered IM to horses diffused to the circulation, was transported into serum, synovial fluid, and was adsorbed by articular cartilage. However, there was no predilection of PSGAG for the articular joints with articular damage. The data indicated that the dose given in this study does have the effect of increasing hyaluronic acid levels in the synovial fluid.

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