Systemic Effects of a Commercial Preparation of Chondroitin Sulfate, Hyaluronic Acid and N-acetyl-D-glucosamine When Administered Parenteral to Healthy Cats

Julia K. Veir*
Craig B. Webb
Michael R. Lappin

Department of Clinical Sciences, Colorado State University
Campus Delivery 1620
Fort Collins, CO 80523
Address correspondence to Dr. Bauer (jbauer@cvm.tamu.edu).
*Corresponding author: jveir@colostate.edu, Fax: 970-297-1275

KEY WORDS: chondroitin sulfate, hyaluronic acid, N-acetyl-D-glucosamine, feline, Glycosaminoglycans

ABSTRACT
Decreased concentrations of glycosaminoglycan (GAGs) have been documented in experimental models of equine osteoarthritis and naturally occurring idiopathic cystitis in both cats and humans. These findings have led to investigations into the efficacy of exogenous supplementation of GAGs in veterinary medicine. A-cyst® is a commercially available veterinary medical product designed for intravesicular administration into the urinary bladder containing 100 mg/mL of chondroitin sulfate and 5 mg/mL of hyaluronic acid in a 10% solution of N-acetyl-D-glucosamine (NAG), a GAG precursor. Administration of A-cyst® to cats at the dose used in this study for this length of time appears to be safe and well tolerated. Some degree of improvement in oxidative stress was demonstrated by a reduction of oxidized glutathione but there may be an increase in the proinflammatory state if the increase in mRNA expression encoding for TNF-alpha and IFN-alpha occurs in diseased animals as well. Down regulation of CD44 expression may have applications in control of tumor metastasis, a non-traditional application of glycosaminoglycans. However, as this study was performed in a small number of healthy animals and overall lymphocyte populations, further more detailed investigations in varying disease states should be pursued.

INTRODUCTION
Proteoglycans are composed of many different glycosaminoglycan (GAG) components. Decreased concentrations of GAGs have been documented in experimental models of equine osteoarthritis (Frisbie 2009) and naturally occurring idiopathic cystitis of both cats (Buffington 1996, Pereira 2004) and humans (Hurst 1987, Parsons 1994). These findings have led to investigations into the...

MATERIALS AND METHODS

A-cyst® is a commercially available veterinary medical product designed for intravesicular administration into the urinary bladder containing 100 mg/mL of chondroitin sulfate and 5 mg/mL of hyaluronic acid in a 10% solution of N-acetyl-D-glucosamine (NAG), a GAG precursor. It has also been administered parenterally in the equine for its anti-inflammatory effects (Frisbie 2009). Intravesicular administration requires at minimum sedation, and in some cases, general anesthesia. In order to use the product for maintenance of remission in inflammatory diseases, an enteral or parenteral route would be preferred. Therefore, the purpose of this study was to examine the safety and systemic anti-inflammatory effects of parenteral administration of A-cyst® to healthy adult cats.

The protocol was approved by the Animal Care and Use Committees of Colorado State University and ArthroDynamic Technologies. Eight healthy 1-year-old domestic short haired cats were included in the study. Prior to inclusion, cats were screened for general health via complete blood count (CBC), biochemical analysis, and urinalysis, and were evaluated for the presence of serum antibodies against feline herpesvirus-1, feline calicivirus, and feline immunodeficiency virus, as well as the presence of feline herpesvirus 1 and calicivirus collected onto pharyngeal swabs by virus isolation. Cats were allowed to acclimate to the facility for 3 weeks prior to the beginning of the study, and fed a commercial maintenance diet free choice. Cats were monitored for attitude, appetite, and activity level, and subjective observations were recorded daily. A-cyst® was administered at a rate of 0.1 mL/lb (0.45 mL/kg) intramuscularly every 4 days for a total of five treatments. Sampling for oxidative stress, lymphocyte populations, and cytokine mRNA expression in blood was done on days 0, 14, 28, and 56. Sampling for CBC, biochemical analysis and urinalysis was done on days 0 and 56.

Effect on oxidative stress was evaluated via measurement of reduced glutathione to oxidized glutathione ratio (GSH:GSSG) and Trolox Equivalent Antioxidant Capacity (TEAC) assay. Glutathione was quantified from EDTA-preserved blood using an enzymatic assay as described previously (Webb 2003). The concentration of GSH was determined by the difference between the total glutathione (GSH+GSSG) concentration and the GSSG concentration. The GSH:GSSG was then calculated. Antioxidant capacity was measured in 10 mL of plasma with a commercially available spectrophotometric assay as described previously in dogs (Hinchcliff 2000).

Evaluation of lymphocyte populations via flow cytometry was performed as follows. Red cells were lysed via ammonium chloride treatment and remaining cells were placed in plates for immunostaining. Non-specific binding was blocked by addition of 10% normal cat serum. Immunostaining was done at 4°C in buffer containing PBS, 0.1% sodium azide and 2% fetal bovine serum.

Cells were immunostained with fluorescein isothiocyanate (FITC) conjugated antibodies to feline CD4 (vpg34) and phycoerythrin-conjugated antibodies to feline CD8 (vpg9) and with a cross-reactive PE/cy5 labeled antibody to CD44 (IM7). Gates for analysis were set on lymphocyte population based on forward and side-scatter. Data were collected using a FACSCalibur cytomter and were analyzed using CellQuest.

Whole blood for determination of mRNA cytokine expression in blood was stored in RNAlater. Blood was collected into EDTA tubes and then mixed with RNAlater solution at a ratio of four parts RNAlater to one part anticoagulated whole blood. Samples were allowed to equilibrate at 40°C for 24 hours prior to storage at -80°C. Samples were thawed in batch at room temperature and RNA extracted using the QIAamp RNA mini kith. The level of mRNA expression for interleukin (IL)-1, IL-
2, IL-4, IL-6, IL-10 and IL-12 and interferon (IFN)-α and tumor necrosis factor (TNF)-α was assessed with quantitative real-time PCR using published assays and TaqMan primer probe (Leutenegger 1999, Kipar 2001). Amplifications were carried out in duplicate in 25 μł reactions containing (final concentration) 12.5 μł Mastermix, i 0.5 μł (400 nM) each primer, 0.2 μł (80 nM) probe, 6.3 μł PCR grade water, and 5 μł template cDNA. Relative quantification was carried out using comparative Ct method (Applied Biosystems 2001) with feline GAPDH as the reference gene and each cat’s pre-treatment sample as the control sample for across time comparisons

**SUMMARY**
Evaluation of statistical significance was first performed on all variables using the repeated measures ANOVA with time as the only fixed effect. For those variables for which time was significant (p<0.05) further comparisons were made between days 0, 14, 28, and 56 and reported as significant if p<0.05.

Subjectively, no change in activity, appetite, or attitude, was noted over the course of the study period. Transient (<24 hours) pain at the site of injection was the only adverse event noted. No significant changes in complete blood count or differentials, biochemical analyses, or urinalyses were detected.

Oxidized glutathione was significantly decreased for time overall (p=0.0088) and at day 28 when compared to baseline (p<0.05). Reduced glutathione and GSH: GSSG were not significantly changed over time (p=0.2681 and 0.4456, respectively). While not statistically significant, TEAC did increase over time (p=0.0514).

The percentage of lymphocytes positive for CD4 was significantly decreased for time overall (p=0.0003), and at days 14 and 56 compared to baseline (p<0.05). The percentage of lymphocytes positive for CD8 did not change significantly over time (p=0.2966). Mean fluorescence intensity of staining of CD44 on lymphocytes decreased over time overall (p=0.0001), and compared to baseline at all time points afterwards (days 14, 28, and 56, p<0.05). Expression of IL-1 was not detectable at any time points. Levels of expression of IL-2, IL-4, IL-6, IL10, and IL-12 did not significantly change over time. Expression of both IFNalpha and TNF-alpha increased over time (p=0.0121 and <0.0001, respectively). Compared to baseline, both IFN-alpha and TNF-alpha expression were significantly increased at days 28 and 56.

**DISCUSSION**
Administration of A-cyst® to cats intramuscularly at the dose used in this study appears to be well tolerated and safe over the course of 8 weeks as demonstrated by lack of clinically detectable ill effects or changes in biochemical parameters.

State of oxidative stress, as manifested by the decreased accumulation of oxidized glutathione, seemed to improve, though reduced glutathione levels did not increase. There are several explanations for this.

- First, glutathione was measured in a mixed cell population as compared to evaluation of levels in individual subsets of lymphocytes. Therefore, changes within subsets may have been overwhelmed by the overall response.
- Second, though depletion of reduced glutathione is the dominant response to an acute rise in reactive oxygen species, in many chronic inflammatory states, total glutathione production increases with a corresponding increase in oxidized glutathione and more stable levels of reduced glutathione (Aukrust 1995).

Therefore, in these cats with no obvious acute inflammatory stimulus the decrease in oxidized glutathione may reflect an improved ability to deal with chronic inflammatory diseases.

The decrease in the percentage of lymphocytes that stained positive for CD4 is an unexpected finding. To the authors’ knowledge, no evaluation of the impact of exogenous GAG supplementation on circulating...

lymphocyte subset in cats exists. However, in mice models of osteoarthritis, no impact on lymphocyte subsets was detected after supplementation with glucosamine (Panicker 2009). CD4+ lymphocytes are the predominant subpopulation of lymphocytes in autoimmune joint diseases in humans (Weyand, 2000), but whether this decrease in CD4+ percentage of lymphocytes in the circulation would be of benefit in more localized systems of inflammation is unknown.

Similarly, the decrease in staining for CD44 in this study was unexpected. CD44 is one of the main receptors for hyaluronic acid on cell surfaces and is well recognized as a signal for lymphocyte activation. This may be of some interest in tumor control in that up regulation of CD44 is associated with increase in metastatic activity (Lesley 1997). Traditionally, the increase in TNF-alpha and IFN-alpha would be associated with a proinflammatory state. There is no published evaluation of the impact on expression of cytokines by peripheral blood mononuclear cells by supplementation of glycosaminoglycans. There is evidence of a dose-dependent increase in expression of mRNA encoding for TNF-alpha when human uterine fibroblasts are cultured with a high concentration of hyaluronic acid (Kobayashi 1997). The impact of this in diseased animals is unknown and merits further study.

CONCLUSION
In conclusion, administration of A-cyst® to cats at this dose for this length of time appears to be safe and well tolerated. Some degree in improvement in oxidative stress was demonstrated by a reduction of oxidized glutathione but there may be an increase in the proinflammatory state if the increase in mRNA expression encoding for TNF-alpha and IFN-alpha occurs in diseased animals as well. Down regulation of CD44 expression may have applications in control of tumor metastasis, a non-traditional application of glycosaminoglycans. However, as this study was performed in a small number of healthy animals and overall lymphocyte populations, further more detailed investigations in varying disease states should be pursued.

REFERENCES


a ArthroDynamic Technologies, Lexington, KY
b Colorado State University Veterinary Diagnostic Laboratory, Fort Collins, CO
c GSH/GSSG-412, OXIS Research, Portland, OR
d Antioxidant Assay Kit, Sigma-Aldrich
e Serotec, Raleigh, NC
f Pharmingen, Franklin Lakes, NJ
g BD Biosciences, San Jose, CA
h Qiagen, Germantown, MD
i Applied Biosystems, Foster City, CA