Measurement of urinary glycosaminoglycans in dogs

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Abstract

Recent work in humans with protein losing nephropathies has revealed increased urine concentrations of sulfated glycosaminoglycans (GAGs). Differences exist between normal patients, those with glomerulonephritis (GN), and those with amyloidosis thus potentially allowing differentiation without a renal biopsy. Aims of this study were to validate a simple spectrophotometric assay used to measure canine urinary GAGs, establish a normal reference range, and determine optimal storage conditions. Urine GAG concentrations were measured in a limited number of dogs with glomerulonephritis or amyloidosis.

Fourteen healthy dogs were placed in metabolic cages and all urine was collected for 24 hours. Serum and urine creatinine concentrations were measured at the beginning and end of the collection period. Urine collected at the beginning of the 24-hr period was centrifuged and the supernatant used to measure a spot GAG concentration and a spot glycosaminoglycan to creatinine ratio (GCR). A well mixed aliquot of the 24-hr sample was centrifuged, the supernatant used to measure the 24-hr total GAG, and stored at 4°C and -20°C for 1, 7, and 30 days. All dogs were used to determine effects of time and temperature (n=14), however, only dogs with an endogenous creatinine clearance > 2 ml/min/kg (n=10) were used to determine normal values. A standard absorption curve using a 1,9-dimethlymethylene blue dye and dilutions of chondroiton-4-sulfate was developed to estimate total GAG concentration. Repeated measures analysis of variance was used to test for effects of storage temperature and time on stability of urinary GAG. A p-value of < 0.05 was considered significant. Relationships between spot urinary GAG concentration, spot urinary GAG to creatinine ratio (GCR) and 24-hr total GAG excretion were estimated using simple linear regression.

Single urine samples were collected by cystocentesis from dogs with GN or renal amyloidosis. The diagnosis was confirmed by clinical evaluation or by histologic analysis. Urine protein, creatinine and GAG concentrations were measured.

There were no time or temperature effects on urine GAG concentrations for up to 1 day at 4°C and 30 days at -20°C. Mean 24-hr total GAG excretion ± standard deviation was 1.586 ± 0.461 mg/kg of body weight. Mean spot GAG concentration and spot GCR were 5.007 ± 1.588 mg/dl and 0.023 ± 0.01 respectively. Neither spot GAG concentration (R^2=0.4216) nor GCR

(R^2= 0.0839) were adequate predictors of 24-hr total GAG. The GCR's from dogs with renal disease were not different from normal dogs.

This study established normal total urinary GAG values in dogs. Contrary to findings in humans, there was no correlation between 24-hr total sulfated GAG and spot GCR in dogs, limiting clinical utility of this test. Further work is needed to determine if either total sulfated GAG or the spot GCR can be used to differentiate causes of protein-losing nephropathies in dogs.

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Table of Contents

List of Tables and Figures

List of Abbreviations

ECC	Endogenous creatinine clearance
GAG	Glycosaminoglycan
GAGs	Glycosaminoglycans
GBM	Glomerular basement membrane
GCR	Glycosaminoglycan to creatinine ratio
UP/C	Urine protein-to-creatinine ratio
GN	Glomerulonephritis
DMMB	1,9-dimethylmethylene blue
C-4-SO4	Chondroitin-4-sulfate
dH2O	Deionized water
UCr	Urine creatinine

I. Introduction

Glycosaminoglycans (GAGs), formerly referred to as mucopolysaccharides, are molecules of repeating disaccharides found in biologic organisms. Glycosaminoglycans are generally found in the form of proteoglycans in integral plasma membrane proteins, connective tissue matrix and in basement membranes.[1] The GAG heparin is found unbound to proteins within mast cell granules. In human urine, GAGs are unbound. Types of glycosaminoglycan (GAG) chains found in human urine are heparan sulfate, dermatan sulfate, keratan sulfate and chondroitin sulfate.[2] Various methods have been used to quantify urine GAG concentrations including precipitation reactions, electrophoresis, thin layer chromatography, and spectrophotometry. With the exception of the latter, these methods are time consuming and technically difficult and in recent years have been used with less frequency in clinical patients. The more commonly used spectrophotometric method is based on a direct ionic interaction of the positively charged dye 1,9 dimethylmethylene blue (DMMB) and the negatively charged sulfate regions of GAG. This binding alters light absorbance of the resulting solution and can thus be used to measure total sulfated GAG concentrations.

Measurement of urinary excretion of GAGs has been done in human and veterinary medicine to evaluate pathogenesis of various diseases and therapies including mucopolysaccharidosis, rheumatoid arthritis, urolithiasis, neoplasia of the urinary tract, extra-corporeal shock wave lithotripsy, diabetes mellitus, interstitial cystitis, renal failure, glomerulonephritis and renal amyloidosis.[3-17] Of particular interest are reports of altered GAG excretion in human patients with glomerular diseases.[3,14,16,18,19] Existing reports of urine GAG measurements in dogs are scarce.[4,11]

There are two main categories of glomerular disease in dogs, glomerulonephritides and renal amyloidosis. Amyloidosis is considered much less common than glomerulonephritis (GN), but because the two have similar clinical findings a renal biopsy is necessary to differentiate them.[20] Renal biopsies can be obtained via surgical, laparoscopic, and percutaneous (with or without ultrasound guidance) techniques. There is risk and expense involved with each of these procedures.[21] In addition, some degree of expertise is required with all techniques. Past perception has been that information gained by obtaining a renal biopsy does not justify risk and cost in dogs. While some reports show an equivocal difference in survival time between dogs with GN and amyloidosis, current veterinary literature contains a greater number of reports of

prolonged survival times and responses to therapy in dogs with GN.[22-28] Therefore, differentiating the two disease processes does provide prognostic information. Additionally, recent advances in management of GN make prospects for these patients even greater compared with those having amyloidosis.[24]

Recent reports by Tencer et al found significant differences in urine GAG-to-creatinine ratios (GCR) from single urine samples of normal human patients and those with primary GN or two different forms of renal amyloidosis.[18,19] Urine GCR in patients with GN and amyloidosis were significantly decreased compared with controls. Conclusions of these reports suggest the DMMB spectrophotometric assay of urine GAG has the ability to distinguish normal patients from those with primary GN and amyloidosis. Based on data provided, it may also be possible to differentiate between the two pathologic states. Contrary to this, however, an earlier report by other investigators found increased urine GCR in humans with GN.[3] Differences in GCR in these studies seem to be due to variation in preparation of buffer solutions, use of different absorption wavelengths, questionable statistical analysis and possible interfering substances.[3,18,19]

Despite these differing results, the possibility of differentiating glomerular diseases without a renal biopsy is intriguing and would be beneficial in veterinary medicine. A GAG assay theoretically may also be useful in diagnosing GN that has reached an end, non-proteinuric stage, in diagnosing non-proteinuric medullary renal amyloidosis, and in monitoring therapies utilizing synthetic GAGs. Additionally, further study of the pathophysiology of urolithiasis and diagnosis of mucopolysaccharidosis may stem from this work.

The purpose of this study is to determine if the DMMB spectrophotometric method can be used to accurately quantify urine GAG in dogs. Additionally, correlation of the GCR from a single urine sample to the 24-hour excretion of GAG and methods of preservation will be determined in normal canine urine.

II. Literature Review

A. Canine Glomerulonephritis

Glomerulonephritis is a common cause of chronic renal disease in dogs.[28] Glomerular injury caused by GN in dogs is immunologically mediated. In humans and dogs, immunoglobulins and complement factors have been shown to be bound to glomerular structures.[23,28-33] There are two mechanisms by which this immunologic damage is initiated: 1) preformed circulating antigen-antibody complexes are deposited or are trapped within glomeruli,

or 2) antigen is trapped in the glomerular capillary wall and circulating antibodies form complexes with them. Formation of anti-glomerular basement membrane antibodies in naturally occurring GN has not been proven in dogs or cats.

Glomerulonephritides are manifested histopathologically as forms of inflammatory or sclerotic lesions of glomeruli. Glomerulonephritis results in urinary protein loss and may lead to chronic renal failure, hypertension, thromboembolism and a shortened survival time.[34-38] Glomerulonephritis can be a primary, idiopathic disorder or it can occur secondary to infectious, inflammatory, immune mediated or neoplastic diseases.[34,39]

Injurious processes that result from immune complexes seem to be dependent on their intraglomerular location. Much of what is known about the pathophysiology is based on *in vitro* studies, but some general mechanisms are noteworthy. Once immune complexes have formed, there is a complex combination of complement activation, neutrophil and macrophage infiltration, platelet aggregation, activation of the coagulation cascade and fibrin deposition. Neutrophils, macrophages and mesangial cells produce oxidants and proteinases in response to immunoglobulins. Platelet activation and aggregation result in eicosanoid (thromboxane and leukotriene) formation and coagulation. Thromboxanes interfere with immune complex disposal, are chemotactic for neutrophils and may decrease glomerular filtration rate through vasoconstriction and mesangial cell contraction.[20] Increased urinary thromboxane excretion has been associated with GN and impaired immune complex clearance in animals.[23,32,40-42] Nitric oxide is released by many cells during glomerular inflammation and can induce cytotoxicity. Platelets, as well as neutrophils, macrophages, endothelial cells and mesangial cells can release platelet-activating factor, which can neutralize negative charges in the glomerular capillary walls and enhance albuminuria.[43] Platelet-activating factor and eicosanoids are chemotactants for neutrophils and macrophages, perpetuating a cycle of inflammatory mediator release.[43] These injurious mediators cause morphologic changes within glomeruli. Mesangial cell and matrix proliferation and glomerular basement membrane (GBM) thickening can occur and with continued injury, glomerulosclerosis may develop. Eventually, irreversible damage to the glomerulus leads to a non-functional nephron.

B. Canine Renal Amyloidosis

Renal amyloidosis is an uncommon cause of renal failure in dogs. It has been reported as familial and reactive in origin in dogs.[26,27,44-49] The kidney is the most common site of amyloid

deposition in dogs.[50] Canine renal amyloid has been identified as analogous with human amyloid-AA, which is composed of amino-terminal fragments of the acute phase reactant serum amyloid-A protein arranged in a â pleated sheet. This protein is produced by the liver in response to interleukin-1, a cytokine released by activated macrophages. The function of serum amyloid-A is unknown. Once the â pleated sheet has formed, proteolysis and dissolution fail to occur. Renal AL amyloid is made up of immunoglobulin light chains. This form has been documented in a single dog with myeloma.[51]

Renal amyloidosis can occur as a primary or secondary disorder.[20,22] Clinicopathologic findings in dogs with renal amyloidosis have been studied.[26,27,44,45] Familial forms in the Beagle, Shar Pei, and Foxhound are associated with medullary deposition of amyloid with lesser involvement of glomeruli. This medullary deposition can lead to papillary necrosis, interstitial fibrosis, lymphoplasmacytic inflammation, tubular dilation, intratubular oxalate crystals and resultant renal failure, either acutely or chronically. In cases seemingly unassociated with a familial predisposition, amyloid primarily deposits in glomeruli. Affected glomeruli are hypocellular and can become sclerotic and atrophied. Mechanisms by which amyloid AA induces these changes are not understood. Renal failure is common even in cases predominated by glomerular deposition.[26]

C. Proteinuria

The greatest amount of proteinuria occurs with renal amyloidosis and membranous glomerulonephritis.[52] Inflammatory and degenerative changes that occur in the kidneys in amyloidosis and GN cause loss or decreased production of heparan sulfate in the GBM.[53] This likely is a result of damage to endothelium, the source of heparan sulfate in the GBM.[54] This leads to a loss of electrostatic and possibly size selectivity and subsequent loss of anions, typically proteins, into the urine. Inflammation need not necessarily be present to decrease sulfated GAG content as it is known that this occurs in isolated diabetic rat GBM.[55]

The urine protein-to-creatinine ratio (UP/C) is a simple test that allows an approximation of urinary protein loss and negates urine concentration as a complicating factor.[20,56-58] While glomerular filtration rate and urine concentration may change, the ratio between urinary creatinine and protein presumably remains constant in most instances. Studies examining healthy and ill dogs utilized correlation coefficients or simple regression to determine correlation between random (spot) UP/C and 24-hr protein excretion expressed as milligrams of protein per

kilogram of body weight. High correlation was found in each case. However, only one of these studies evaluated more than one spot sample and no attempts were made to randomize time of day at which these were collected. Thus, it is not truly known if variation in the UP/C occurs throughout the day in dogs.

D. Glycosaminoglycans

1. Structure and Function

A GAG is a linear carbohydrate molecule. There are five major families of GAGs, based predominantly on their structure: heparin and the heparan sulfates, chondroitin sulfates, dermatan sulfates, keratan sulfates, and hyaluronate. Keratan sulfates are repeating sequences of hexosaminyl and galactosaminyl residues while the others are hexosaminyl and uronyl residues. Sulfated GAGs are negatively charged as a result of the carboxylate terminals and sulfate moieties. With the exception of the urinary tract, GAGs are not found as free molecules but rather are bound to proteins as proteoglycans. In humans, urine GAGs are two-thirds chondroitin sulfate with the balance mainly heparan sulfate.[2]

Little is known of the function of GAGs in the urinary tract and even less is known of the function of free urinary GAGs. Within the kidney, they are produced by glomerular endothelial cells.[54] Glycosaminoglycans are believed to be a key component in the permselectivity of the glomerulus as a result of their high density of negative charges. Increased permeability of the GBM to anionic ferritin has been demonstrated after removal of heparan sulfate by enzyme digestion.[53] Removal of GAGs from urothelium by acid digestion decreases defense against bacterial and crystalline adherence, whereas replacement restores this activity.[59,60] Numerous *in vitro* studies have demonstrated inhibition of calcium oxalate crystal formation by addition of synthetic GAGs to urine.[59] Use of exogenous GAGs in humans for prevention or treatment of calcium oxalate uroliths in recurrent formers decreases recurrence rate and number of uroliths.[59] Intravesicular administration of pentosan polysulfate, a synthetic sulfated GAG, provides relief in many humans with interstitial cystitis.[60] Combined with decreased urinary GAG excretion in these patients, this is supportive evidence of GAGs as a urothelial protectant.[61] However, in more recent years increased GAG concentrations have been found in patients with interstitial cystitis.[13,62,63] The reasons for these contradictory findings are not clear and emphasizes the relative lack of knowledge of GAG function and poor understanding of the pathophysiology of interstitial cystitis.

2. Urinary Glycosaminoglycan Research

Urinary GAGs have been studied to determine their role in numerous disease processes in humans. Alterations in urinary GAG excretion has been investigated in humans with mucopolysaccharidoses, rheumatoid arthritis, systemic lupus erythematosus, urinary bladder neoplasia, and bacterial lower urinary tract infection.[5-10,64-66] Urinary GAGs have also been evaluated in numerous human nephropathies including diabetic nephropathy, glomerulonephritides, and amyloidosis.[16-19] The veterinary literature is barren of research about the significance of urinary GAGs; reports include measurement in cats with an interstitial cystitis-like syndrome, in a family of dachshunds with mucopolysaccharidosis IIIA, and in dogs undergoing extracorporeal shock wave lithotripsy.[4,11,12]

Of direct importance to the research at hand are reports of altered renal excretion of GAGs in humans with glomerulonephritides or amyloidosis. Tencer et al reported on 150 cases of GN in humans, 63 normal individuals, and 19 individuals with diabetic nephropathy.[18] Individuals with any form of GN or diabetic nephropathy had significantly decreased median urinary GAGs, expressed per mmol of creatinine, (1.98 mg/mmol and 1.17 mg/mmol respectively) compared with normal individuals (2.87 mg/mmol).[18] This finding was attributed to decreased GAG synthesis in damaged nephrons. In another series, the same author found similar results with a median GCR of 0.21 mg/mmol in AA amyloidosis, 0.33 mg/mmol in AL amyloidosis, 1.73 mg/mmol in primary glomerulonephritides, and 2.67 mg/mmol in normal individuals.[19] Decreased GAG synthesis was again cited as a cause for decreased GCR. Interestingly, the GCR for all three groups were significantly different from each other potentially allowing the GCR to act as a screening test to separate them.

Similar studies have also found alterations in GCR in human patients with glomerulonephritides.[3,14] Mitsuhashi et al reported on 55 individuals with various forms of GN and 14 healthy controls.[3] The GCR of individuals with IgA nephropathy (0.031), membranous GN (0.041), and minimal change GN (0.038) were significantly greater than healthy controls (0.018). Increase in GAG excretion is likely renal in origin rather than due to systemic production as serum GAG concentrations are not increased in humans with IgA nephropathy.[14] This is also supported by information that humans in renal failure caused by primary GN actually have decreased serum GAG concentrations.[14] Additionally, as would be expected, serum and urinary GAGs do not correlate.[14] Obviously the results of these studies contrast with those by

Tencer et al. Tencer et al proposed their finding of decreased GCR was due to methodological differences, namely that they measured decrease in absorbance at 590 nm instead of increase at 520 nm. This was done because 590 nm lies within a separate photopeak of DMMB. This still fails to explain the contrary findings of decreased and increased GAGs between these two as the decrease in absorbance at 590 nm still should have correlated linearly with increasing concentrations of GAG. It would seem that other differences in methodology are more likely to explain the difference. Tencer et al modified the methods of Mitsuhashi et al by using smaller volumes, 96-well microtitre plates, a urinary preservative, and a sodium acetate buffer of a dramatically different pH.

There is conflicting information as to whether the GCR corrects the urine GAG concentration for variable states of dehydration or approximates 24-hr total GAG excretion. One study claimed that spot urinary GCR showed good linear correlation with 24-hr GAG excretion, therefore the GCR from a single sample could be used for comparing patients.[66] Mitsuhashi et al, employing the GAG assay used in the current study, claim that daily and circadian urinary excretions of GAG were studied in 5 humans and that excretion rates were constant.[3] However, when urinary GAGs are estimated by measuring hexuronic acid concentration or uronic acid concentration, non-parallelism in the daily fluctuation of GAGs and creatinine occurs.[64,67] Not only does the GCR fluctuate throughout the day, it also fluctuates from day to day. However, the GCR from the total urine sample collected over 24 hours is consistent from day to day.[67] Numerous methods have been used to quantitate total urinary GAGs. These include electrophoretic and various precipitation based tests.[68] These methods can be time consuming, require multiple processing steps, and are predominantly used in research laboratories. Alternatively, non-precipitated, direct urine samples can be used with addition of polyanionic dyes such as Alcian Blue or 1,9, DMMB.[3,5,6,8,10,14] The result is binding of the dye to GAGs and a corresponding colorimetric change measurable with a spectrophotometer. This latter method is much less time consuming and in humans has been shown to correlate with measurement of hexuronic acid content, via the uronic acid carbazole test, which seems to be the gold standard.[5,8]

E. Spectrophotometry

Spectrophotometry is the branch of chemistry that deals with the identification of chemical compounds by measuring the absorbance or transmission of light of varying wavelengths through a solution containing the compound of interest. Therefore,

spectrophotometry can be used quantitatively and qualitatively to assess light-absorbing species and its properties are theoretically described by *Beer's Law:* A = log Po/P = åbc where A is absorbance, or optical density, and represents the log of the power of incident light (Po) divided by the power of emergent light (P). This fraction is also known as transmittance or molar extinction coefficient. Absorbance is directly proportional to molar absorptivity (å), path length of the light in centimeters (b), and concentration of the absorbing substance in moles per liter. Each chemical compound has a unique absorption pattern when considered over a range of wavelengths of light. This pattern is known as a spectrum and is typically measured over a wavelength range from 200 to 800 nm. Light wavelengths in the range from 200 to 320 nm are in the ultraviolet region whereas those from 320 to 800 nm fall in the visible spectrum of light. By scanning a given compound over this wavelength range, characteristic absorption peaks and troughs will result, which allow for its specific identification.

When considering the study of a solute in liquid solution, non-absorbing factors such as scatter and reflection of light energy coupled with absorbing factors such as interaction of light with solvents decrease the transmittance through a substance. These sources of decreased transmittance are accounted for by subtracting their value and that of the absorbance of the solvent from that caused by light energy interactions with the solute of interest. The resulting value is the absorbance of the solution.

The excitation of valence electrons about an atom or molecule to higher energy orbitals, along with rotational and vibrational energies of organic molecules in solution, are additional sources of energy consumption, although to a lesser extent. The energy consumed in these processes accounts for part of the decrease in energy of the emergent light that is of measurable interest.

Spectrophotometry can be used to quantify the concentration of an organic substance within a solution based on the properties described above. Initially the wavelength of light at which the greatest absorbance of a standard dye solution occurs is determined. This is known as the peak absorbance or lambda max (λ max). Absorbance of subsequent dye solutions to which a solute has been added is altered and is directly proportional to concentration of the solute in the solution. Dimethylmethlene blue is a metachromatic dye frequently used in this manner for quantifying urinary GAGs. Once a standard curve relating absorbance to incrementally increasing concentrations of a solute is generated, the concentration of that solute in subsequent

solutions can be determined via linear regression, as long as the two solutions vary only by the concentration of the solute.

III. Goals

There are four goals of the current study. 1) Determine if the DMMB spectrophotometric assay can be used to accurately quantify urine GAG in normal dogs. 2) Determine correlation of spot urine GCR or GAG concentration to 24-hr GAG content and determine normal values. 3) Determine appropriate storage conditions that will not alter urine GAG measurement. 4) Evaluate urine GAG concentration and GCR in dogs with renal disease and compare them with values from normal dogs.

IV. Materials and Methods

A. Chemicals and Solutions

The method used to determine total sulfated GAGs in canine urine was adapted from the human literature.[3] This method was chosen because it is unaffected by proteinuria, a problem noted with other versions of the DMMB-based GAG assay. Chondroitin-4-sulfate (C-4-SO4) was used as a representative sulfated GAG to approximate total sulfated GAG. Specifics of this assay are described as follows. Ten milligrams (mg) of (C-4-SO4) were added to 100 ml of deionized water (dH2O) to create a 10.0 mg/dl solution. The solution was mixed with a magnetic stirrer for 10 minutes and stored at room temperature in a light-protected brown glass bottle with an air-tight cap. The same stock solution was used for all stages of testing. Aliquots of stock solution were placed in individual test tubes and diluted appropriately to prepare concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5, and 10.0 mg/dl for use in determining a standard curve (Figure 4.1).

Table 4.1 C-4-SO4 Standard Solution Volumes

Concentration (mg/dL) of C-4-SO4	Volume of dH2O (ml)	Volume of 100 mg/L C-4-SO4 (ml)
0.0	2	0
0.25	1.95	0.05
0.5	1.9	0.1
0.75	1.85	0.15
1.0	1.8	0.2
1.5	1.7	0.3
2.0	1.6	0.4
3.0	1.4	0.6
4.0	1.2	0.8
5.0	1	1
7.5	0.5	1.5
10.0	0	2

A dye solution of DMMB was prepared by adding 11 mg of DMMB to 1 L of 0.05 M sodium acetate buffer. A 0.05 M sodium acetate solution was made by adding 6.8 g of sodium acetate trihydrate to approximately 990 ml of dH2O and mixed with a magnetic stirrer for 15 minutes, when all visible crystals were dissolved. The solution was adjusted to a pH of 4.75 by dropwise addition of 0.1 M hydrochloric acid or 1M sodium hydroxide. Sufficient dH2O was added to reach a final volume of 1 L. This stock DMMB solution was stored at room temperature in a light-protected brown glass bottle with an air-tight cap. Additional stock solution was prepared as needed and was kept no longer than 1 month. No observable precipitate was noted at any time.

B. Instrumentation

A UV-Visible Beckman DU640B spectrophotometer was used for determination of change in absorbance of C-4-SO4 stock solutions and urine samples. Disposable cuvettes with a 1-cm length of light path and 4.5-ml volume were used. Change in absorbance of 2.5 ml of stock DMMB solution using dH2O as a blank was determined in triplicate at 350, 400, 450, 500,520, 535, 550, 600, 650, and 700 nm each day. This was done to assure stability of the dye solution.

To assess linearity over a wide range of concentrations, a standard curve for C-4-SO4 was determined using concentrations of 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5, and 10.0 mg/dl. This was done by adding 250 µl of the respective concentration of C-4-SO4 to 2.5 ml of DMMB. Each cuvette was inverted twice to mix, and change in absorbance was read within 1 minute. Each dilution was measured in triplicate. To determine if change in absorbance occurred over time, C-4-SO4 concentrations of 0.0, 0.25, 1.0, 4.0, and 10.0 mg/dl were measured in triplicate at 1, 2, 3, 4, 5, 10, 20, and 30 minutes. No obvious significant change occurred.

All urine samples were processed by centrifuging them at 2700 rpm for 5 minutes and then removing the supernatant for further use. Change in absorbance caused by each urine sample was measured in triplicate at 520 nm, one of the absorbance peaks of the DMMB- C-4-SO4 complex. This was done by adding 250 µl of supernatant to 2.5 ml of DMMB solution in each cuvette, inverting twice, and measuring change in absorbance within 1 minute. Glycosaminoglycan concentration was then calculated with use of the regression equation for the standard curve generated each day.

The pH of the sodium acetate buffer was measured utilizing an ATI Orion Model 310 perpHect Log R meter with a Thermo Orion perpHect gel-epoxy triode model 9207BN probe. This meter has a sensitivity of 0.01 pH.

Urine creatinine concentration was measured from the supernatant of urine after having been centrifuged at 2700 rpm for 5 minutes. Urine was submitted to the clinical pathology laboratory of the Virginia-Maryland Regional College of Veterinary Medicine, where an Olympus AU 400 Automated Chemistry Analyzer utilizing the modified Jaffe procedure was used to measure creatinine concentration. Quality control and calibration procedures are performed in this laboratory every 7 days.

C. Procedures

To assess stability of GAG and creatinine in canine urine, ten dogs of various breeds, weights and sexual status were selected and determined to be healthy based on physical examination, urine specific gravity and urine dipsticks that estimate urine protein, bilirubin, glucose, ketone, and blood concentrations. This will be referred to as storage trial 1. Urine was collected by untimed free catch and the change in absorbance of the supernatant was measured within 4 hours of collection. A single creatinine concentration was measured in each sample. Five to 20-ml aliquots of urine were placed into polyethylene vessels and stored at 4^{o} C and -20^{o}

C for 1 day and 30 days, at which time they were brought to room temperature using a water bath set at 23^{o} C. Aliquots were then mixed for 1 minute using a Lab-Line multiwrist shaker. The GAG and creatinine concentrations of each aliquot were then calculated and measured, respectively, as described previously.

Nine additional urine samples, from different dogs than used in trial 1, were studied at the same temperatures for 1 day and 7 days to evaluate GAG stability at an intermediate time and to assess a filtration method. This will be referred to as storage trial 2. Free catch urine samples were processed, stored, rewarmed, and measured as previously described. Attempts to filter urine through 0.45 and 0.9 micron syringe end filters (Pall Acrodisc 25 mm Syringe Filters) were made to improve removal of urine sediment. This resulted in complete obstruction to flow after approximately 3-5 ml of urine was filtered. Due to expense of these filters and poor success, their use was discontinued. Gravitational filtering was then attempted using medium grade, 9-cm filter paper (Fisher Scientific). It required approximately 15 minutes to filter 5-10 ml of urine. This method was used for the urine samples in this storage trial.

A third trial, storage trial 3, was conducted using fourteen of the sixteen dogs that made up the 24-hr urine collection study population described below. Fifty milliliters of well-mixed urine from the 24-hr total urine volume for each patient were collected. The samples were processed, stored, rewarmed, and measured as previously described except no filtration was performed. Creatinine and GAG concentrations were measured within 4 hours of the end of the 24-hr collection and after 1, 7, and 30 days of storage at 4^{o} C and –20^{o} C.

Twenty-four-hour urine collections were performed to determine normal GAG excretion. Inclusion criteria for entry into the study were normal results of the following tests: physical examination, complete blood count, biochemical profile, *Dirofilaria immitis* ELISA, urinalysis, urine protein-to-creatinine ratio (UPC), and aerobic urine culture. Stainless steel cages (3 ft x 3ft x 3 ft) equipped with underlying collection pans were used. Standard mesh screening with approximately 2mm holes was stretched across a wooden frame and placed under the slatted steel flooring and above the collection pan as an added barrier to fecal contamination of urine. Heavy plastic was placed over the lower 75% of the cage door to prevent dogs from urinating through the cage door. Eight French, 48-inch long sterile suction tubing was attached to the drainage port of the urine collection pan at one end and to a polyethylene collection bottle held at a dependent level.

All dogs received 0.01-0.02 mg/kg of acepromazine intramuscularly 15-20 minutes prior to the following procedures. All dogs were weighed and then given 5% of their body weight in water via orogastric tube. The urinary bladder was then catheterized with an 8 or 10 French red rubber urinary catheter and urine was removed via suction with a 60-ml syringe. Once the flow of urine ceased, manual palpation of the bladder was done to insure it was empty and a final attempt was made to suction urine with the syringe. Spot GAG and creatinine concentrations were determined on this sample within 4 hours. Three milliliters of blood were collected into 5-ml lithium heparin anticoagulated glass tubes via jugular venipuncture. This was used to determine plasma concentrations of glucose, urea nitrogen, creatinine, phosphorus, calcium, total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase, total bilirubin, cholesterol, sodium, potassium, chloride, and total carbon dioxide. Dogs were then placed within the cages described above for 24 hours. Collection bottles were emptied every 2 hours, and urine for each dog was placed in a separate container and stored at 4^{o} C. Water was offered 12 hours into the collection but was not consumed or spilled by any of the dogs. Food was not offered to avoid contamination of the urine by spilled food. At the end of the 24-hour period all dogs were weighed, given 0.01-0.02 mg/kg of acepromazine intravenously and the urinary bladder catheterized and expressed as described above. Acepromazine was administered intravenously in this case to rapidly tranquilize the dogs and catheterize them so as not to risk loss of urine due to urination outside of the metabolic cages. Urine obtained by catheterization was added to the 24-hour total urine collection. Three milliliters of blood was collected for determination of creatinine. Total 24-hour urine volume, urine creatinine, protein, and GAG concentrations were measured on a well-mixed aliquot of this urine within 4 hours. A 50-ml, well mixed aliquot was separated for the storage study as described previously.

Endogenous creatinine clearance (ECC) was calculated using the equation:

ECC= ([UCr] x Urine Volume) ÷ ([Average Serum Cr] x collection time x Average Body Weight)

Where [UCr]= Urine creatinine concentration, Urine Volume= volume of urine collected in 24-hours, [Average Serum Cr]= average concentration of the serum creatinine (mg/dl) at the beginning and end of the 24-hour collection, Collection time= exact number of minutes in the collection period, Average Body Weight = average of the body weights (kg) at the beginning and end of the 24-hour collection.

Data from all dogs that completed the 24-hour urine collection were used for the storage study. Only data from dogs with an ECC > 2 ml/min/kg were used for determination of normal spot GAG, spot GCR, and 24-hour GAG content values.[69,70] Spot GCR and 24-hour GAG content were determined using the equations:

24-hour GAG content= [GAG] x Urine Volume

GCR= spot [GAG] ÷ [Urine Cr]

where [GAG] is the concentration of GAG, urine volume is the total volume collected during this 24-hour period, and urine creatinine is that measured at the beginning of the collection period.

Five dogs with renal disease were evaluated. Each dog was determined to have significant proteinuria (UPC >2) or a clinical condition consistent with glomerular disease. Diagnostic evaluation of these dogs was performed as deemed appropriate by its attending veterinarian. Diagnostics performed in all dogs included a complete blood count, a biochemical profile including the same parameters previously mentioned, a urinalysis, aerobic urine culture, and UP/C. Three of the five had histologic evaluation of a renal biopsy to confirm the specific form of glomerular disease. A complete list of the diagnostic tests performed on each dog can be seen in Appendix B.

D. Statistical Analysis

Each day that urine was assayed a standard curve using C-4-SO4 concentrations of 1.0, 2.0, 4.0, 7.5 and 10.0 mg/dl and simple linear regression analysis were performed to determine the relationship between change in absorption and C-4-SO4 concentration. Regression lines were assessed for linearity and a coefficient of determination was calculated using Microsoft Excel 2000 software. Coefficients of determination greater than 0.98 were considered strong evidence of a linear relationship and the corresponding regression equations were used to estimate sulfated GAG concentration from the change in absorbance of each urine sample.

Data from the storage studies were analyzed for effects of time and temperature on GAG and creatinine concentrations using a complete randomized block design. The difference between the GAG concentration at time 0 and at each time/temperature combination, denoted as dGAG, was used as the variable for analysis. Direct analysis of the GAG concentration would be inappropriate since the sample taken at time 0 had not undergone any treatment (time or temperature effect), whereas dGAG incorporates both the initial sample and the treatment

effects. Repeated measures analysis of variance was performed using The SAS System software, version 8.2 (SAS Institute Inc, Cary, NC 27513) to evaluate the hypotheses:

$$dGAG_4{}^{o}{}_{C,\ 1\ day} = [\ GAG_0] - [\ GAG_4{}^{o}{}_{C,\ 1\ day}] = 0$$

$$dGAG_4{}^{o}{}_{C,\ 7\ days} = [GAG_0] - [GAG_4{}^{o}{}_{C,\ 7\ days}] = 0$$

$$dGAG_4{}^{o}{}_{C,\ 30\ days} = [GAG_0] - [GAG_4{}^{o}{}_{C,\ 30\ days}] = 0$$

$$dGAG_{-20}{}^{o}{}_{C,\ 1\ day} = [GAG_0] - [GAG_{-20}{}^{o}{}_{C,\ 1\ day}] = 0$$

$$dGAG_{-20}{}^{o}{}_{C,\ 7\ days} = [GAG_0] - [GAG_{-20}{}^{o}{}_{C,\ 7\ days}] = 0$$

$$dGAG_{-20}{}^{o}{}_{C\ ,\ 30\ days} = [GAG_0] - [GAG_{-20}{}^{o}{}_{C\ ,\ 30\ days}] = 0$$

Urine creatinine was analyzed in the same manner, with the same hypotheses, using dUCr as the analytical variable. A p-value of < 0.05 was considered significant, thus rejecting the above hypotheses. Simple linear regression analysis was used to determine the relationship of 24-hr GAG content (mg/kg of body weight) and spot GCR, and 24-hr GAG content and spot GAG concentration. Coefficients of determination were calculated, using Microsoft Excel 2000 software, as an estimate of the relationship between these variables. Values for spot GCR, spot GAG concentration, and 24-hr GAG content were assessed for normality using histograms and were subsequently expressed as means with standard deviations.

V. Results

A. Urine Glycosaminoglycan and Creatinine Stability

Variation in the change in absorbance of the DMMB and in the slope of the standard curve were used to rapidly assess quality control on a daily basis. Variation was minimal as shown in Figure 5.1 with a coefficient of determination greater than 0.98 for each curve. The DMMB solution initially was blue; however, as increasing concentrations of C-4-SO4 were used the resulting solutions became increasingly intense shades of violet and then pink.

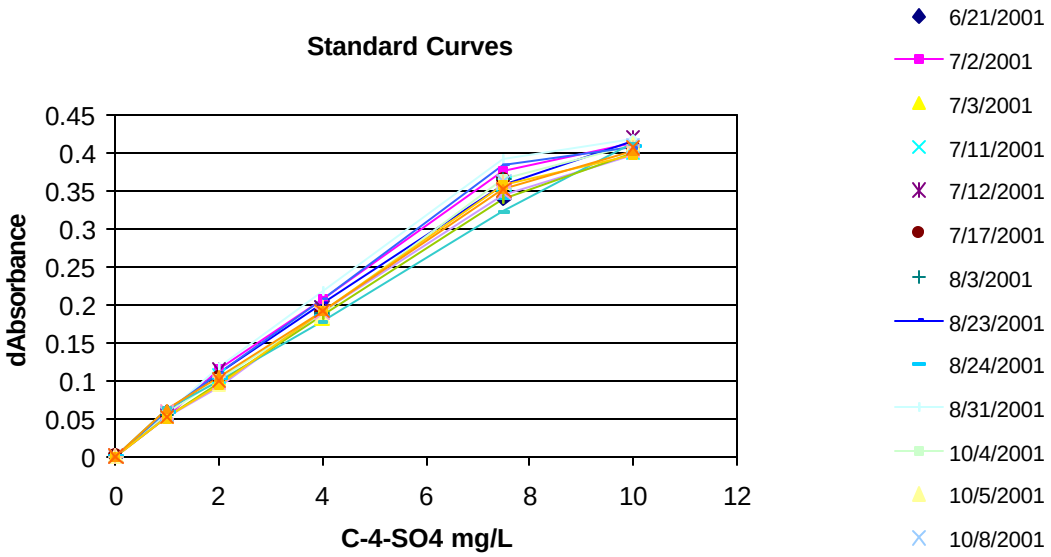

Figure 5.1 Change in light absorbance of increasing concentrations of chondroitin-4-sulfate (C-4-SO4).

Storage trial 1 revealed GAG concentrations were not significantly different at 4° C or -20° C after 1 day but were different at day 30 ($p = 0.0023$ at 4° C, $p = 0.002$ at -20° C) (Figure 5.2).

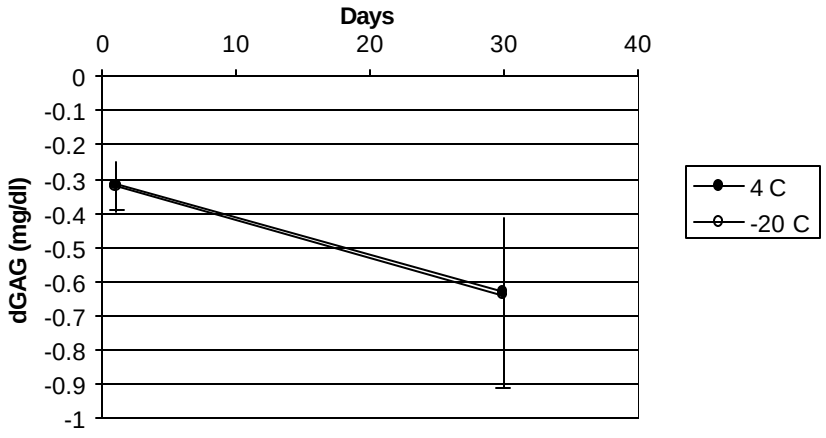

Figure 5.2 Storage trial 1. Mean ± SE of dGAG (the difference between urine GAG concentration between time zero and 1 and 30 days) at 4° C and –20° C (n=10 for each point).

The dGAG decreased from baseline, indicating increased GAG concentrations through 30 days. On day 1 and 30, the mean GAG and dGAG ± standard deviation were: 3.91 mg/dl and -0.318 ±

0.074 mg/dl, and 4.22 mg/dl and -0.63 ± 0.282 mg/dl at 4°C; 3.91 mg/dl and -0.322 ± 0.072 mg/dl, 4.23 mg/dl and -0.640 ± 0.225 mg/dl at -20° C, respectively.

Urine creatinine values were not significantly different from baseline when stored at -20° C for 1 and 30 days or when stored at 4° C for 1 day (Figure 5.3).

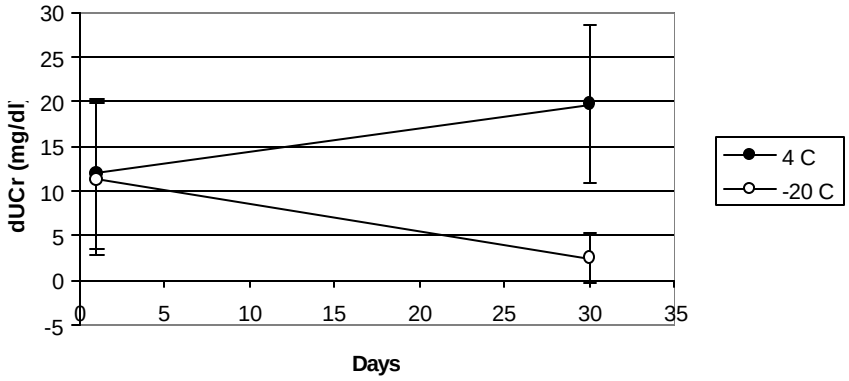

Figure 5.3 Storage trial 1. Mean ± SE of dUCr (the difference in urine creatinine concentration (mg/dL) at time zero and 1 and 30 days) at 4° C and –20° C (n=10 for each point).

Initially dUCr increased, indicating decreased creatinine concentrations, at both storage temperatures. At -20° C, mean UCr was 267.1 mg/dl and dUCr was 11.3 ± 8.52 mg/dl after 1 day, but when measured on day 30, mean UCr was 275.8 and dUCr was only 2.52 ± 2.73 mg/dl. Similarly, at 4° C, mean UCr was 266.4 mg/dl and dUCr increased by a mean of 12.0 ± 8.36 mg/dl after 1 day, but then after 30 days mean UCr was 258.6 mg/dl and dGAG became significantly different from initial values with a mean of 19.7 ± 8.78 mg/dl (p= 0.014).

Storage trial 2 revealed GAG concentrations were not significantly different at -20° C on day 7 but were different at 4° C on day 1 (p= 0.0002) and day 7 (p= 0.025) and at -20° C on day 1 (p= 0.002) (Figure 5.4).

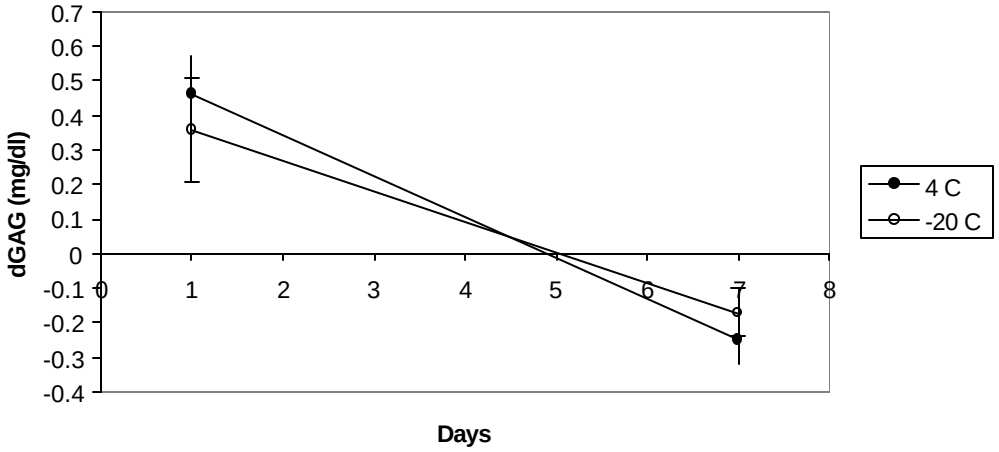

Figure 5.4 Storage trial 2. Mean ± SE of dGAG (the difference between urine GAG concentration between time zero and 1 and 7 days) at 4° C and –20° C (n=9 for each point).

In storage trial 2, mean GAG and dGAG ± standard deviation was 3.66 mg/dl and 0.46 ± 0.108 mg/dl at 4^{o} C, and 3.77 mg/dl and 0.358 ± 0.15 mg/dl at -20^{o} C on day 1. On day 7, these values were 4.37 and -0.25± 0.071 mg/dl at 4^{o} C and 4.29 and -0.17 ± 0.07 mg/dl at -20^{o} C.

Storage trial 3 revealed no significant difference in the urine GAG concentration at either 4^{o} C or -20^{o} C on day 1 or on day 30 at -20^{o} C, but values were significantly different at all other time and temperature combinations. Urine GAG values fluctuated similarly at both storage temperatures as shown in Figure 5.5.

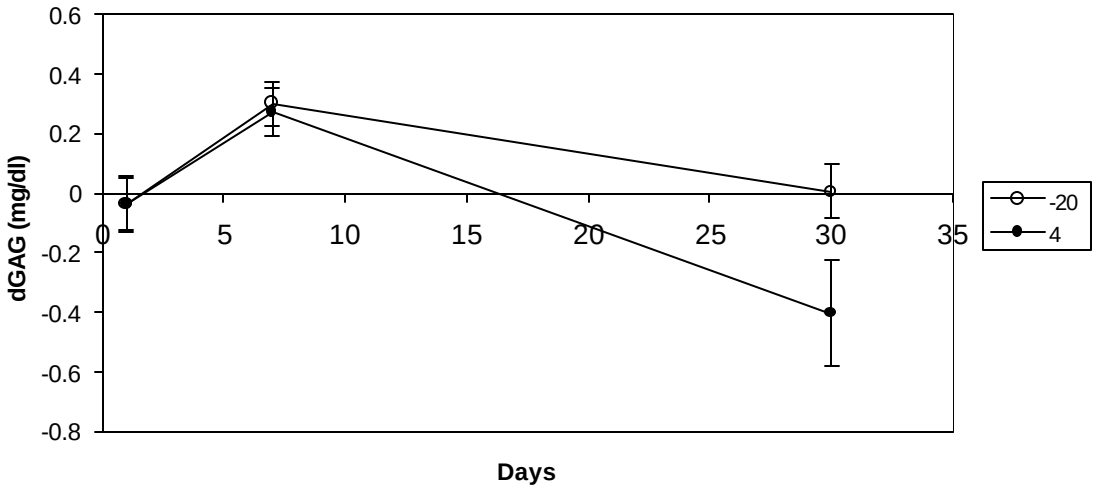

Figure 5.5 Storage trial 3. Mean ± SE of dGAG (after 1, 7, and 30 days) at 4° C and –20° C (n=14 for each point).

Mean urine GAG and dGAG ± standard deviation were: on day 1, 3.86 mg/dl and -0.037 ± 0.089 mg/dl at 4° C and 3.86 mg/dl and -0.037 ± 0.093 mg/dl at -20° C; on day 7, 3.55 mg/dl and 0.27 ± 0.081 mg/dl at 4° C and 3.52 mg/dl and 0.30 ± 0.074 mg/dl at -20° C; on day 30, 4.22 mg/dl and -0.404 ± 0.178 mg/dl at 4° C and 3.82 mg/dl and 0.045 ± 0.091 mg/dl at -20° C.

B. Glycosaminoglycan Excretion

1. Normal Dogs

Sixteen dogs met inclusion criteria for this study. Thirteen were mixed breeds, two were blue tick coonhounds and one was a Siberian husky. Of these sixteen dogs, fourteen participated in the 24-hour urine collection. The urine collection system of dog 535, a male mixed breed, leaked an excessive amount during the first several hours of the collection. This dog was extremely nervous and agitated within its cage and at approximately 12 hours into the collection seized for approximately 20 seconds. This dog and its data were thus eliminated from the study. Dog 515, a female spayed mixed breed, was used to practice the 24-hour collection procedures approximately 1 week before data collection was to begin on all dogs. This dog was very difficult to catheterize and thus urinary tract trauma was a concern. Additionally there was concern she had become dehydrated as evidenced by a urine output of only 4.73 ml/kg/24 hours. Therefore she was eliminated from the study. Of the fourteen dogs that completed the 24-hour urine collections, 10 had ECC values greater than 2 ml/min/kg with a mean ± SD of 2.581 ± 0.352 ml/kg/min. This group consisted of six spayed females, three intact males and one

neutered male. The mean body weight was 21.57 kg. The age of each dog was unknown, but all were adults.

Mean 24-hour GAG content ± standard deviation was 1.586 ± 0.461 mg/kg. Mean GCR from the 24-hr total urine sample was 0.044 ± 0.012. Mean ± standard deviation of spot GAG concentration and spot GCR were 5.007 ± 1.588 mg/dl and 0.023 ± 0.01 respectively. No significant linear relationship could be demonstrated between either spot GAG and 24-hour GAG content or spot GCR and 24-hr GAG content (Fig. 5.6-5.7).

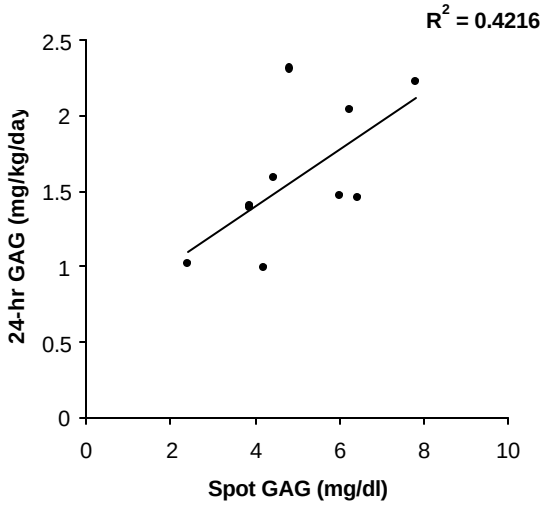

Figure 5.6 24-hour total GAG versus spot urine GAG concentration. Each point represents one dog.

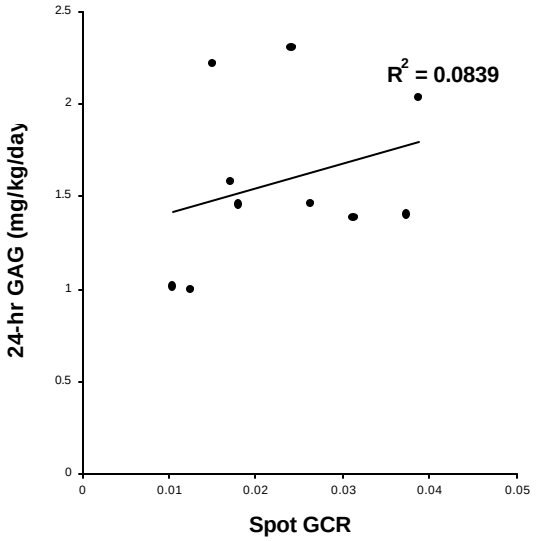

Figure 5.7 24-hour GAG versus spot GCR. Each point represents one dog.

Therefore neither spot GAG concentration (R^2=0.4216) nor GCR (R^2= 0.0839) were adequate predictors of 24-hr total GAG in this study.

The GCR of the 24-hr total urine sample was consistently greater than that of the spot sample (Figure 5.8). Both urine creatinine and urine GAG concentrations tended to be less in the 24-hr total urine samples than in the spot samples with creatinine decreasing with greater magnitude (Figures 5.9 –5.11). Additional data for the dogs used for the 24-hr urine collections can be found in Appendix A.

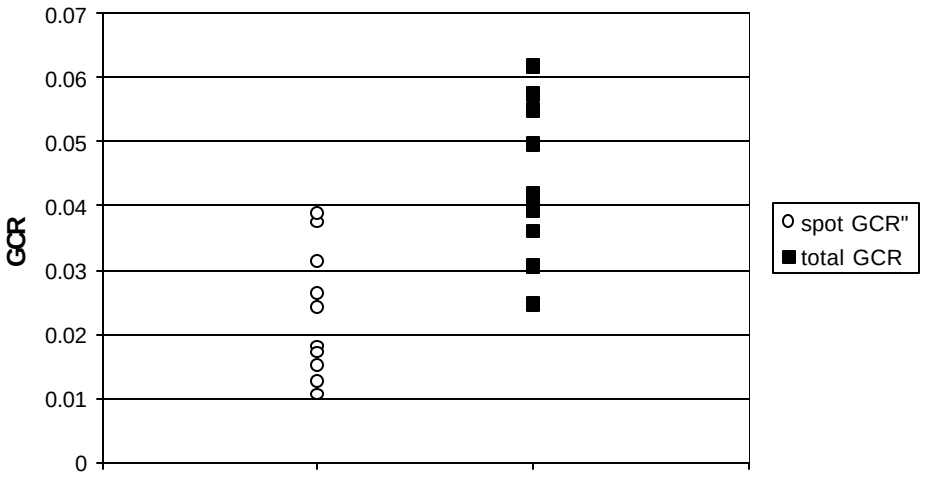

Figure 5.8 Total and spot GCR values for 10 dogs.

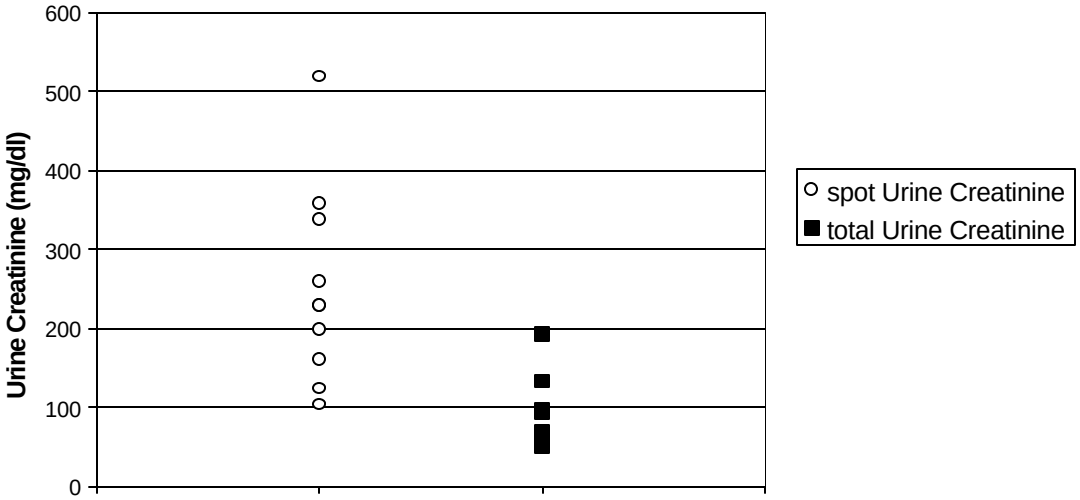

Figure 5.9 Spot and 24-hr total urine sample creatinine concentrations for 10 dogs.

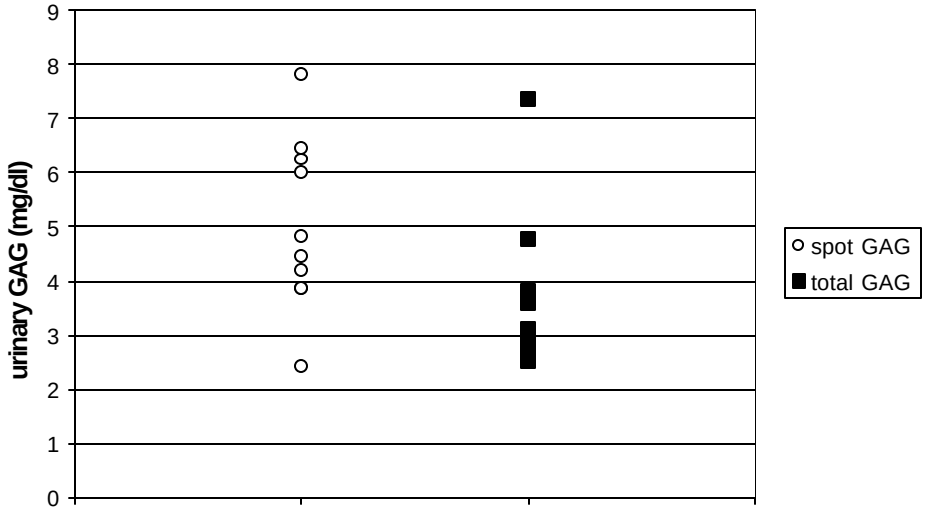

Figure 5.10 Spot and 24-hr total urine sample GAG concentrations for 10 dogs.

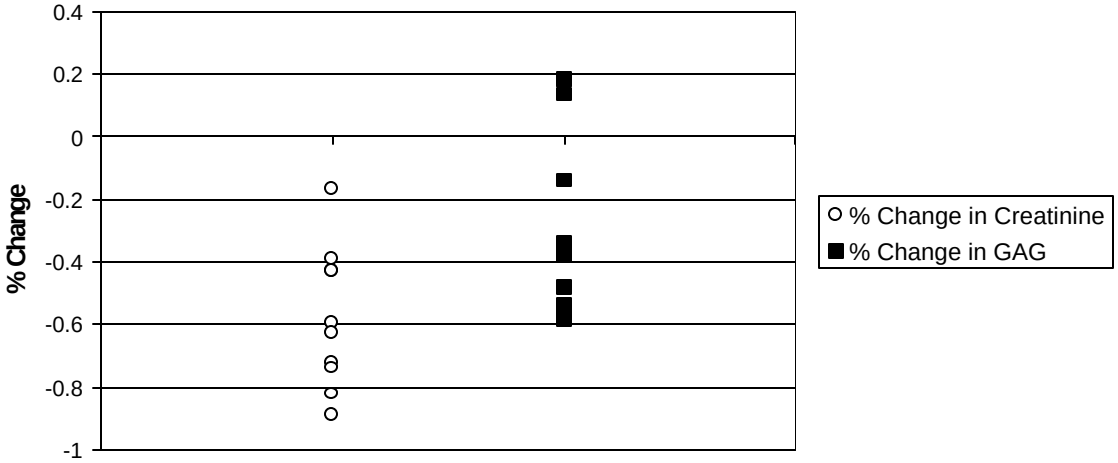

Figure 5.11 Change in urine creatinine and GAG concentrations as a percent of the spot value for 10 dogs. (% Change = (spot – 24-hr total) ¸ spot).

2. Dogs with Renal Disease

A limited number of dogs with protein losing nephropathies, GN or renal amyloidosis were evaluated and their urinary GAG and creatinine concentrations measured on single, untimed samples. Their complete data are summarized in Appendix B. Dog # 68080 was a 13-year-old Labrador retriever with mesangioproliferative GN. The dog also had pituitary dependent hyperadrenocorticism. This dog had a UP/C of 10.62 and a GCR of 0.022. Dog

#67060 was a 2.5-year-old Shar Pei with renal amyloidosis and chronic renal failure. This dog had a UP/C of 0.15 and a GCR of 0.033. Dog # 66654 was an 8.5-year-old brittany spaniel in chronic renal failure with proteinuria. This was clinically determined to be consistent with end-stage GN; however, renal biopsy was not done. This dog had a UP/C of 4.27 and a GCR of 0.044. Dog # 66190 was a 10-year-old pomeranian with chronic GN and hypertension. This dog had a UP/C of 11.6 and a GCR of 0.037. Dog # 68814 was a 9-year-old German shepherd with membranoproliferative GN, chronic renal failure and bacterial endocarditis. This dog had a UP/C of 2.71 and a GCR of 0.022. The mean and standard deviation of the GCR for all dogs was 0.03 ± 0.009.

VI. Discussion

A. Urine Glycosaminoglycan and Creatinine Stability

The standard curve was linear over the range of 0 to 0.75 mg/dl as expected based on reports in humans.[3] The dye and G-4-SO4 solutions underwent color changes as expected. The mean spot GAG concentrations and GCR values in the 10 dogs completing the 24-hr urine collections were of similar magnitude to values reported in normal and abnormal humans using various methods of GAG measurement.[3,9,10,18,19,65,66] These findings support that the assay accurately measured free sulfated urinary GAGs in urine of normal dogs.

Results of the storage trials revealed one contradictory finding regarding urine GAG stability. In storage trials 1 and 3, dGAG initially decreased at day 1, whereas in storage trial 2 it increased. There are several differences in how these studies were performed, which may partially account for these results. In storage trials 1 and 2, dogs were not screened for illness by any means other than a urine dipstick and specific gravity. Storage trial 2 differed from the others in that a filtration method was used to remove as much sediment from the urine as possible. This was abandoned as it significantly slowed processing of the samples and caused loss of urine. Additionally, it did not seem to diminish the amount of sediment visually observed when frozen samples were defrosted, which was the reason for using it. Exactly how the filter paper may have altered the results is not apparent, but when comparing storage trials 2 and 3 at 1 and 7 days, they are opposite in variation of dGAG. This raises concern that the filter paper had some effect. Samples were carefully maintained at 4^{o} C or -20^{o} C, aliquots were stored in separate vials such that repeated defrosting was not needed and defrosting was done slowly in a warm water bath set below body temperature. Therefore, heat stress seems unlikely as a cause of

the decrement of GAG concentrations at day 1 in storage trial 2. These differences in procedure do not logically explain the contradictory findings on day 1, but raise concern that storage trial 2 was flawed.

Results from storage trial 3 seem likely to be the most accurate for several reasons. Most importantly, my experience level and attention to methodology improved over time such that by storage trial 3, I had processed in excess of a thousand samples. Storage trial 3 differed from the other 2 in that the dogs were screened carefully for disease, especially of the urinary tract, and their urine was collected over 24-hrs and refrigerated during this time. The decrease in dGAG on day 1 in storage trial 3 was trivial at 0.9% of the baseline value. Additionally, the dGAG values in storage trial 3 were more precise as can be seen by evaluation of the standard error of the means for dGAG (Fig. 5.5).

There were many similarities in the results of the storage trials. In all 3 storage trials GAG concentrations were not significantly different from the time 0 samples after 1 day at either 4^{o} C or -20^{o} C. This appears to be the case in humans as well, even when urine is held at room temperature.[3] The dGAG consistently decreased, indicating that GAG concentrations had increased, when measured at 30 days of storage at 4^{o} C. The cause for this increase in GAGs is not apparent. Bacterial growth was considered as a source of GAG production. However, this seems unlikely since bacterial urinary tract infection does not affect GAG concentrations in humans and bacteria would not be expected to grow at 4^{o} C.[66] The effect of storage times and temperatures on urinary GAGs has not been reported, thus the existing scientific literature does not provide other explanations. Using storage trial 3 as the most reliable results the conclusion should be made that urine GAG concentrations remain constant for only 24 hours at 4^{o} C or less.

Urine creatinine concentrations appear to remain stable for 30 days when stored at -20^{o} C. This was expected as the references provided in the Olympus AU400 Analyzer manual report creatinine is stable indefinitely at -20^{o} C. This should be considered the ideal long-term storage temperature with 4^{o} C being an acceptable alternative for 24 hours.

B. Glycosaminoglycan Excretion

Perhaps the most important finding in this research is that the spot GCR does not correlate with the 24-hr total GAG. This correlation is important because if GCR estimates the 24-hr GAG content it could be used as a convenient, single sample for comparing GAG excretion amongst individuals. If GCR cannot be used, a 24-hr urine collection would be

necessary. This is generally impractical in canine patients without the use of an indwelling urinary catheter. The effects of catheterization on GAG concentrations are unknown and technical support to monitor the collection process is needed, making this approach impractical for most clinical patients.

Several possible causes for lack of correlation between the spot and 24-hr samples seem reasonable to consider. The physical differences between the spot sample and 24-hr GAG content are that the latter was passed through the cage and suction tubing. The effects of dander, hair, and interactions with the tubing on GAG and creatinine concentrations are not known. All spot GCR samples were collected in the morning. Lack of correlation would occur if the excretion of GAG or creatinine are not constant, especially if there is non-parallelism in their fluctuation through the day. In humans there is conflicting information as to whether the GCR corrects the GAG concentration for variable states of dehydration or approximates 24-hr GAG content.[3,64,66,67] Nearly all reports measuring urinary GAGs that are pertinent to the current research express them as a ratio with urinary creatinine. One of these claims that random sample urinary GAG excretion showed good linear correlation with 24-hr GAG content, therefore the GCR from a single sample could be used for comparing patients.[66] Mitsuhashi et al, using the GAG assay in this study, claim that daily and circadian urinary excretions of GAG were studied in 5 humans and that excretion rates were constant.[3] However, when urinary GAGs are estimated by measuring hexuronic acid or uronic acid concentration, non-parallelism in the daily fluctuation of GAGs and creatinine occurs.[64,67] Not only does the GCR fluctuate throughout the day, it also fluctuates from day to day. However, the GCR from the 24-hr urine sample is consistent from day to day.[67] The accuracy of this article should be questioned as it utilized only 3 individuals. In the current study, the spot GCR taken on dogs in the morning was lower than the GCR from the total 24 hr. sample in all 10 dogs. This implies that excretion of GAGs and/or creatinine is not constant. This indeed is the case as both urine GAG and creatinine concentrations fluctuated, with creatinine concentrations decreasing in greater magnitude than urine GAG (Figure 5.9-5.11). The effects of hydration status on GAG production, excretion, or reabsorption have not been reported in humans or dogs. The most plausible cause for decreased concentrations in the 24-hour total urine samples is dilution caused by administration of 5% of body weight of water at the beginning of the 24-hr collection period, immediately following collection of the spot sample. This was necessary, as two dogs used in trial runs did not drink for

24 hours and lost weight, implying dehydration. This administration of water in a large, single bolus certainly is not a normal situation and may have been the cause for the lack of correlation between spot GCR and 24-hr total GAG excretion. However, in retrospect this seems to have been a reasonable, although very simple, test to determine parallelism of urine GAG and creatinine concentrations with the conclusion that they do not parallel each other.

Dogs in the current study were fed the day prior to the 24-hr urine collection, but not during the collection, which raises concern about the effect of feeding on urine GAG and creatinine excretion. Urine creatinine is not affected by the fed or non-fed state, but whether urinary GAG concentration is affected by feeding is unknown.[69]

Differences in how GAGs and creatinine enter the urine could be reasons for the non-parallel fluctuation. Creatinine undergoes glomerular filtration without any significant tubular secretion or reabsorption. It is not know what percentage of urinary GAGs arise from serum, what percent arise from the kidneys and urinary tract, or if GAGs undergo tubular secretion or reabsorption. Additionally purported sources of urinary GAGs are the glomerular endothelium, mesangial matrix, and the uroepithelium of the bladder. Each of these is either within the glomerular filter or distal to it. Therefore GAGs entering urine from these locations would not be undergoing the same filtration/elimination as creatinine and therefore may account for non-parallel fluctuation.

Fluctuation in the GAG and creatinine concentrations could be investigated by measuring urinary GAG and creatinine concentrations at numerous times throughout a 24-hr collection. The effects that fasting and altered hydration status had could be determined by acclimating the dogs, as originally planned, such that they would eat and drink normally during the collection period. However, at this point, it must be concluded that spot sample GCR is not an adequate predictor of 24-hr GAG content and does not correct for variable states of hydration in the dog. Additionally, one must question the validity of using spot GCR to compare urinary GAG excretion in humans and in cats with interstitial cystitis.

Originally a study goal was to compare spot GCR in normal dogs and dogs with idiopathic renal amyloidosis or idiopathic GN confirmed by renal histology. It was difficult to gather information needed in dogs suspected of having these diseases, thus dogs with any form of glomerular disease were included. This of course fails to eliminate the underlying or concurrent diseases in these cases as a cause for altered GAG concentrations, if present. Mean

GCR and GAG concentrations of the 5 dogs with glomerular diseases were not substantially different from that of the 10 normal dogs, and there was significant overlap in values. Because GCR and GAG concentrations do not seem to correlate with 24-hr GAG content, it is not possible to say whether a difference exists between these groups. Based on the results of the current study, use of 24-hr urine collections would be needed to compare these groups at this point.

VII. References

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VIII. Appendices

A. Data for Normal Dogs

DOG NUMBER	1	2	3	4
Sex	FS	FS	FS	FS
Weight (kg) – Start	20.1	20.8	25.3	21.9
Weight (kg) – End	19.8	20	24.8	21.3
Avg. Weight (kg)	20.0	20.4	25.1	21.6
Total Collection Minutes	1465	1442	1440	1434
Ace (mg/kg)- start of 24 hours	0.02	0.02	0.02	0.02
Ace (mg/kg)- end of 24 hours	0.02	0.02	0.02	0.04
Total urine volume in 24 hours (ml)	949	1576	1386	1326
Urine Production (ml/kg/day)	47.57	77.25	55.33	61.39
Serum Cr.- start of 24 hours (mg/dl)	0.8	0.9	0.9	1.1
Serum Cr.- end of 24 hours (mg/dl)	0.8	1	0.9	1.1
Average Serum Cr. (mg/dl)	0.8	0.95	0.9	1.1
Urine Cr.- spot (mg/dl)	228.6	199	103.5	518.2
Urine Protein- 24 hour sample (mg/dl)	3.5	3	8.5	5.3
Urine Cr.- 24 hours sample (mg/dl)	63.1	52.1	62.9	58.3
ECC (ml/min/kg)	2.56	2.94	2.69	2.27
UPC- 24 hour sample	0.06	0.06	0.14	0.09
Urine GAG (mg/dl)- 24 hour sample	3.13	2.99	2.53	3.60
Urine GAG (mg/dl)- spot sample	6.00	4.81	3.87	7.80
Urine GCR- 24 hour sample	0.05	0.06	0.04	0.06
Urine GCR- spot sample	0.03	0.02	0.04	0.02
% Change in GAG	-0.48	-0.38	-0.35	-0.54
%Change in urine Creatinine	-0.72	-0.74	-0.39	-0.89
Urine GAG total (mg/day)	29.69	47.20	35.12	47.75
Urine GAG (mg/kg/day)	1.46	2.31	1.40	2.22
Urine Protein (mg/kg/day)	1.64	2.31	4.70	3.27

DOG NUMBER	5	6	7	8
Sex	FS	FS	M	M
Weight (kg) – Start	23.2	16	27.7	19.2
Weight (kg) – End	22.5	16	27.8	18.7
Avg. Weight (kg)	22.9	16.0	27.8	19.0
Total Collection Minutes	1440	1414	1410	1403
Ace (mg/kg)- start of 24 hours	0.05	0.02	0.02	0.04
Ace (mg/kg)- end of 24 hours	0.05	0.02	0.02	0.04
Total urine volume in 24 hours (ml)	1240	856	565.5	509
Urine Production (ml/kg/day)	54.27	53.50	20.38	26.86
Serum Cr.- start of 24 hours (mg/dl)	0.9	0.8	1.2	1.1
Serum Cr.- end of 24 hours (mg/dl)	0.9	0.7	1.2	1.1
Average Serum Cr. (mg/dl)	0.9	0.75	1.2	1.1
Urine Cr.- spot (mg/dl)	123.5	356.8	336.9	160.7
Urine Protein- 24 hour sample (mg/dl)	4.9	4.8	32.8	15.9
Urine Cr.- 24 hours sample (mg/dl)	70.6	63.8	193	133.9
ECC (ml/min/kg)	2.96	3.22	2.32	2.33
UPC- 24 hour sample	0.07	0.08	0.17	0.12
Urine GAG (mg/dl)- 24 hour sample	2.55	2.67	4.77	7.37
Urine GAG (mg/dl)- spot sample	3.87	6.43	4.19	6.24
Urine GCR- 24 hour sample	0.04	0.04	0.02	0.06
Urine GCR- spot sample	0.03	0.02	0.01	0.04
% Change in GAG	-0.34	-0.58	0.14	0.18
%Change in urine Creatinine	-0.43	-0.82	-0.43	-0.17
Urine GAG total (mg/day)	31.62	22.88	26.97	37.52
Urine GAG (mg/kg/day)	1.38	1.46	0.99	2.03
Urine Protein (mg/kg/day)	2.66	2.62	6.83	4.38

DOG NUMBER	9	10
Sex	M	MN
Weight (kg) - Start	23.8	17.7
Weight (kg) – End	23.5	16.7
Avg. Weight (kg)	23.7	17.2
Total Collection Minutes	1440	1411
Ace (mg/kg)- start of 24 hours	0.02	0.02
Ace (mg/kg)- end of 24 hours	0.02	0.02
Total urine volume in 24 hours (ml)	838	698.5
Urine Production (ml/kg/day)	35.43	40.61
Serum Cr.- start of 24 hours (mg/dl)	1.1	1.3
Serum Cr.- end of 24 hours (mg/dl)	0.9	1.2
Average Serum Cr. (mg/dl)	1	1.25
Urine Cr.- spot (mg/dl)	229.5	259.8
Urine Protein- 24 hour sample (mg/dl)	4.5	21.6
Urine Cr.- 24 hours sample (mg/dl)	93.2	97
ECC (ml/min/kg)	2.29	2.23
UPC- 24 hour sample	0.05	0.22
Urine GAG (mg/dl)- 24 hour sample	2.86	3.82
Urine GAG (mg/dl)- spot sample	2.41	4.44
Urine GCR- 24 hour sample	0.03	0.04
Urine GCR- spot sample	0.01	0.02
% Change in GAG	0.19	-0.14
%Change in urine Creatinine	-0.59	-0.63
Urine GAG total (mg/day)	23.98	26.71
Urine GAG (mg/kg/day)	1.01	1.58
Urine Protein (mg/kg/day)	1.59	8.95

	AVG.	SD
Weight (kg) - Start	21.57	3.552792329
Weight (kg) - End	21.11	3.657093564
Avg. Weight (kg)	21.34	3.601218929
Total Collection Minutes	1429.9	19.52462832
Ace (mg/kg)- start of 24 hours	0.023889667	0.011001
Ace (mg/kg)- end of 24 hours	0.026304181	0.011902369
Total urine volume in 24 hours (ml)	994.4	367.3764191
Urine Production (ml/kg/day)	47.25914185	16.91621254
Serum Cr.- start of 24 hours (mg/dl)	1.01	0.172884033
Serum Cr.- end of 24 hours (mg/dl)	0.98	0.168654809
Average Serum Cr. (mg/dl)	0.995	0.165747465
Urine Cr.- spot (mg/dl)	251.65	124.6667135
Urine Protein- 24 hour sample (mg/dl)	10.48	9.920775059
Urine Cr.- 24 hours sample (mg/dl)	88.79	44.14990751
ECC (ml/min/kg)	2.580997956	0.352084476
UPC- 24 hour sample	0.104339071	0.057183818
Urine GAG (mg/dl)- 24 hour sample	3.630431508	1.485329234
Urine GAG (mg/dl)- spot sample	5.006635459	1.587871335
Urine GCR- 24 hour sample	0.043698605	0.012017986
Urine GCR- spot sample	0.023107435	0.010155267
% Change in GAG	-0.229935132	0.300882467
%Change in urine Creatinine	-0.580589283	0.224403699
Urine GAG total (mg/day)	32.94150892	8.922864653
Urine GAG (mg/kg/day)	1.585763548	0.46087595
Urine Protein (mg/kg/day)	3.895181928	2.392337442

B. Data for Dogs with Renal Disease

	67060	66654	66190	66814	68080	Normals
RBC (×106)	8.68	1.93	8.78	6.56	4.88	5.5-8.6
HgB (gm/dl)	19	5	19.3	15.6	12.2	13.0-20.1
PCV (%)	55.9	13.9	56.8	47.3	34.7	37.3-62.0
nRBC/100 WBC	0	0	4	0	0	
Reticulocytes	Na	0.8	Na	na	na	
WBC ($\times 10^3$)	8.5	7.8	17.2	8.2	6.7	5.4-16.6
Segs	6.2	6.5	11.3	5.9	5	3.24-10.7
Bands	0	0	0.3	0	0	0-0.25
Lymphs	0.6	0.8	2.9	1.2	1.4	0.75-5.65
Monos	0.7	0.3	0.8	0.7	0.2	0-1.11
Eos	1	0.2	1.7	0.5	0	0.36-2.37
Baso	0	0	0	0	0	0-0.19
Platelets	308	362	374	245	792	179-473
Glucose	89	116	113	83	89	89-135
BUN	23	197	27	37	18	8-27
Creatinine	1.7	15.3	2.1	2.6	0.7	0.6-1.4
Phosphorus	3.2	13.4	3.9	3.6	3	2.6-6.0
Calcium	10.7	11.6	11.8	9.7	11.3	9.5-11.6
Total Protein	6.6	5.5	7	6.7	7.8	5.4-7.2
Albumin	3.3	2.4	2.6	2	3.8	2.7-3.8
Globulin	3.3	3.1	4.4	4.7	4	2.2-4.0
ALT	26	38	62	29	243	13-88

Alk Phos	154	11	101	29	4610	14-105
Total Biliribin	0.2	0.3	0.1	0.2	0.2	0-0.3
Cholesterol	222	284	286	292	248	122-360
Sodium	150	152	148	143	143	144-150
Potassium	3.9	5	4	4.8	4.7	3.4-4.6
Chloride	114	114	116	114	109	108-118
TC02	20	16	18	18	22	16-33
Specific Gravity	1.009	1.010	1.010	1.019	1.041	>1.030
PH	7	5	6	7.5	6	5-7
Protein	trace	3+	4+	3+	3+	0-trace
Glucose	negative	negative	negative	negative	negative	negative
Ketones	negative	negative	negative	negative	negative	negative
Bilirubin	negative	negative	negative	negative	negative	negative
RBC/hpf	35-40	5-8	0	5-6	50-60	0-5
WBC/hpf	1-3	0	0-2	2-4	0-2	0-5
Casts	0	0	0		0	0
Protein:Creatinine	0.15	4.27	11.6	1.6	9.7	<1
Urine Culture	negative	negative	negative		negative	negative
Echocardiogram	NSF			mitral endocarditis		
Renal Biopsy	Yes	No	Yes	yes	yes	
Histologic diagnosis	amyloidosis		membranous GN glomerulosclerosis	mesangio-proliferative GN	mesangio-proliferative GN glomerulosclerosis	
Heartworm test	negative	negative	negative	negative	negative	
Systolic blood pressure	160		240		170	<180

Thoracic radiographs			NSF	NSF		
Abdominal ultrasound	NSF		hyperechoic renal cortices	NSF	bilateral adrenomegaly	
Clinical abnormality	recurrent proteinuria	renal failure	proteinuria	renal failure	weight loss	
Lyme IgM			256			<512
Lyme IgG			2048-vaccinated			<256
R. ricketsii IgM			<8			<16
R. ricketsii IgG			<64			<64
E. canis IgG			<64			<64
Concurrent disease	Shar Pei Fever			endocarditis	hyperadrenocorticism	

Vita

David Clark Grant

Personal

Date of birth: March 16, 1973

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Parents: Richard and Elaine Grant

Wife: Elizabeth Joyce Grant

Academic Appointments

2003-2004	Clinical Instructor, Small Animal Internal Medicine, Department of Small Animal Clinical Sciences, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2002-2003	Chief Resident, Small Animal Internal Medicine, Department of Small Animal Clinical Sciences, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2000-2002	Resident, Small Animal Internal Medicine, Department of Small Animal Clinical Sciences, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
1999-2000	Intern, Small Animal Medicine and Surgery, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida

Education

2000-present	Master of Veterinary Medical Sciences Student, Virginia Polytechnic Institute and State University
1995-1999	Doctor of Veterinary Medicine, University of Florida
1991-1994	Bachelor of Arts, Chemistry, Summa Cum Laude, Virginia Polytechnic Institute and State University

Research Experience

2000-present Master of Veterinary Medical Sciences Research: Evaluation of a spectrophotometric method for measurement of urinary glycosaminoglycans in dogs. Committee Members: S. Dru Forrester, DVM, MS, Dip. ACVIM; David L. Panciera, DVM, MS, Dip. ACVIM; J. Blair Meldrum, DVM, PhD

1998 Individual Investigation: Persistent Soft Tissue uptake of Tc^{99} MDP in Equine Limbs. Advisors: Gregory D. Roberts, DVM, MS, Dip. ACVR and Lisa Neuwirth, DVM, MS, Dip. ACVR

Publications

2002 Wood B.C., Grant D.C., McKlveen T. What's Your Diagnosis: Osteochondroma in a cat. JAVMA 221(7) pp. 939-940.

2001 Grant D.C., Forrester S.D. Glomerulonephritis in Dogs and Cats: Diagnosis and Management. Compendium on Continuing Education for the Practicing Veterinarian. 23(9) pp. 798-805.

2001 Grant D.C., Forrester S.D. Glomerulonephritis in Dogs and Cats: Glomerular function, Pathophysiology, and Clinical Signs. Compendium on Continuing Education for the Practicing Veterinarian 23(8) pp. 739-746.

2001 Schaer M., Halling K.B., Collins K.E., Grant D.C. Combined hyponatremia and hyperkalemia mimicking acute hypoadrenocorticism in three pregnant dogs. JAVMA 218(6) pp. 897-899.

Awards/Honors

2003 Bente Flatland Resident Award

1998 Phi Zeta Veterinary Honor Society

1995 Phi Beta Kappa Honor Society

Professional Organizations

1999-present American Veterinary Medical Association

1998-present Phi Zeta Veterinary Honor Society

Lectures

2003	Measurement of Canine Urinary Glycosaminoglycans, 21st Annual American College of Veterinary Internal Medicine Forum, Charlotte, NC
2002	Nasal Aspergillosis: How We Treat It! A review of the population at the VMRCVM, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2002	Canine Urinary Glycosaminoglycans, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2001	Urinary Glycosaminoglycans: Literature Review and Research Protocol, Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2000	Glomerulonephritis: Diagnosis and Current Treatment, College of Veterinary Medicine, University of Florida
1999	Total Parenteral Nutrition for Veterinary Technicians, College of Veterinary Medicine, University of Florida

Continuing Education

2002	American College of Veterinary Internal Medicine Conference, Dallas, TX
2002	Drug Laws Demystified (Self Assessment Packet), Tampa, FL
2001	North American Veterinary Conference, Orlando, FL

Instructional Activities

2003	Feline lymphoma, leukemia, and leukemia virus. Third year student lectures. Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2002	Instructor, Gastrointestinal Endoscopy: The Basic Level. Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2001	Instructor, Gastrointestinal Endoscopy: The Basic Level. (Spring and Fall) Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech
2001	Instructor, Basic Ultrasound Shortcourse. (Spring and Fall) Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech