

Effects of a urolith prevention diet on urine compositions of glycosaminoglycans, Tamm-Horsfall glycoprotein, and nephrocalcin in cats with calcium oxalate urolithiasis

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Objective—To evaluate urine concentrations of glycosaminoglycans, Tamm-Horsfall glycoprotein, and nephrocalcin in cats fed a diet formulated to prevent calcium oxalate uroliths.

Animals—10 cats with calcium oxalate urolithiasis.

Procedures—In a previous study conducted in accordance with a balanced crossover design, cats were sequentially fed 2 diets (the diet each cat was consuming prior to urolith detection and a diet formulated to prevent calcium oxalate uroliths). Each diet was fed for 8 weeks. At the end of each 8-week period, a 72-hour urine sample was collected. Concentrations of glycosaminoglycans, Tamm-Horsfall glycoprotein, and the 4 isoforms of nephrocalcin in urine samples collected during that previous study were measured in the study reported here.

Results—Diet had no effect on the quantity of Tamm-Horsfall glycoprotein and nephrocalcin in urine. However, the urine concentration of glycosaminoglycans was significantly higher during consumption of the urolith prevention diet.

Conclusions and Clinical Relevance—Feeding a urolith prevention diet increased the urine concentration of glycosaminoglycans, which are glycoprotein inhibitors of growth and aggregation of calcium oxalate crystals. (*Am J Vet Res* 2012;73:447–451)

The prevalence of patients with CaOx urolithiasis is increasing in humans¹ as well as in many other mammalian species, including cats.² In the mid-1980s, CaOx uroliths were rarely recognized in cats. More recently, CaOx uroliths accounted for approximately 40% of submissions to centers that analyze uroliths obtained from cats.^{3,4} Therefore, understanding risk factors for urolith formation is important to mitigate the increasing disease prevalence and to develop strategies to reduce the incidence of uroliths.

Two approaches have been used to investigate mechanisms of urolith formation. One is the physicochemical approach in which investigators assess the degree of supersaturation as a method of predicting the propensity for crystal nucleation.⁵ The physicochemical properties of urine are determined by measuring concentrations of calculogenic analytes and entering them into a computer program that calculates solubility products.⁶ The other

| ABBREVIATIONS | |
|---------------|----------------------------|
| CaOx | Calcium oxalate |
| DEAE | Diethylaminoethyl |
| mS | Millisiemens |
| THP | Tamm-Horsfall glycoprotein |

approach is through biochemical evaluations that identify and characterize organic matrices in urine that influence crystal nucleation and growth. Glycosaminoglycans,⁷ THP,⁸ nephrocalcin,⁹ uropontin,¹⁰ and prothrombin fragment 1¹¹ are common, natural inhibitors of CaOx formation identified in the urine of humans.

In another study¹² conducted by our research group, a diet was formulated to prevent CaOx uroliths in cats. When fed to cats with CaOx urolithiasis, that diet resulted in a significant reduction in urine saturation for CaOx.¹² We hypothesized that this urolith prevention diet altered the magnitude of several organic matrices that influence CaOx urolith formation. To test this hypothesis in the study reported here, concentrations of glycosaminoglycans, THP, and nephrocalcin were measured in urine samples obtained during the aforementioned study¹² conducted by our research group.

Materials and Methods

Animals—Ten client-owned cats with CaOx urolithiasis were evaluated in a study¹² conducted in 2003.

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Mineral composition of uroliths was determined by use of optical crystallography, and only cats with uroliths composed of 100% CaOx were included in that study.¹² These cats included 8 mixed-breed cats, 1 Persian, and 1 Scottish Fold. Mean \pm SD age was 7.7 ± 4.7 years (range, 2.7 to 15.1 years), and mean body weight was 5.77 ± 2.0 kg (range, 3.3 to 11.2 kg). There were 8 castrated males and 2 spayed females. All cats were enrolled in the study approximately 2 weeks after removal of uroliths from the bladder because of clinically active disease. At the time of entry into the study, hypercalcemia, renal failure, bacterial urinary tract infection, or uroliths were not detected in any cat via serum biochemical analyses, urinalyses, aerobic bacterial culture of urine samples, and survey abdominal radiography. Cats were excluded from the study if they had received glucocorticoids or calcium-supplement-type nutritional products within the 2 years preceding the study.

Cats lived with their owners, except during two 72-hour periods for urine collection. During urine collection, cats were housed at the University Of Minnesota College Of Veterinary Medicine Veterinary Medical Center and cared for in accordance with principles outlined by the Animal Care and Use Committee of the University of Minnesota. Written consent authorizing participation of cats in the study was obtained from each client.

Experimental design—Two diets were evaluated (a diet^a formulated to prevent CaOx urolith formation and the diet^{b-h} that each cat was consuming prior to urolith detection).¹² To minimize differences attributable to specific cats in that study,¹² a balanced, first-order crossover design was conducted by use of 2 treatment sequences (diet consumed prior to urolith detection followed by the urolith prevention diet, or the urolith prevention diet followed by the diet consumed prior to urolith detection).¹³ In the aforementioned study,¹² cats were randomly assigned to a treatment sequence. After consuming 1 diet for 8 weeks, cats were placed in cages with special litter pans that allowed collection of urine separately from feces. Voided urine was collected from urine reservoirs every 6 hours for 72 hours and stored in capped containers at 4°C. During urine collections, cats were provided unlimited quantities of food and water, which were replaced twice each day. At the end of the first urine collection period, the alternate diet was fed for 8 weeks, which was followed by collection of a subsequent 72-hour urine sample. In the study reported here, we measured the concentrations of glycosaminoglycans, THP, and nephrocalcin in the same urine samples collected in the aforementioned study.¹²

Measurement of organic matrices—Protein concentration was determined by use of the micro-Lowry method¹⁴ with Folin-Ciocalteu reagent.ⁱ A 10- μ L aliquot of each urine sample was mixed with an equal amount of 15% trichloroacetic acid and incubated for 30 minutes. After incubation, the mixture was centrifuged. Precipitate was dissolved in 1 mL of Lowry reagent containing 0.01% CuSO₄, 0.02% sodium-potassium tartrate, and 0.04% Na₂CO₃ in 0.1N NaOH. Then, 0.1 mL of Folin-Ciocalteu reagent (diluted 2-fold by the addition of water) was added. Samples were incu-

bated for 30 minutes at 24°C. After incubation, the color intensity was measured with a spectrophotometer^l at 750 nm. An aqueous solution of bovine serum albumin (1 mg/mL) was used to construct a calibration standard for concentrations of 10, 20, 30, 40, and 50 μ g/mL.

Urine concentrations of glycosaminoglycans were determined by use of a method described elsewhere.¹⁵ Urine (400 μ L) was mixed with 1.6 mL of 0.05% Alcian blue 8GX aqueous solution containing 50mM MgCl₂; the solution was adjusted to pH 5.8 and stored overnight at 4°C. The next morning, precipitated complex was washed twice with absolute ethanol and then dissolved in 1 mL of 7.5% SDS aqueous solution. Glycosaminoglycan content was determined by measurement of the color intensity on a spectrophotometer at 620 nm. Chondroitin sulfate Aⁱ aqueous solution was used to construct a calibration standard for concentrations of 25, 50, 75, and 100 μ g/mL. This method was used to quantitate the total glycosaminoglycan content of urine.

A method involving the use of diatomaceous earth to isolate THP described in another study¹⁶ allowed nephrocalcin to remain in the supernatant in the study reported here. A 50-mL aliquot of urine was used for separation and measurement of THP and nephrocalcin contents. Isolated THP content was measured on a spectrophotometer at an absorption of 278 nm by use of an extinction coefficient of 10.8. Nephrocalcin isoforms in supernatant were separated by use of a DEAE cellulose column (2 \times 15 cm). Nephrocalcin isoforms were separated by use of a NaCl linear gradient from 0.1 to 0.4M. Eluents of 1.5 mL were sequentially collected. Aliquots (20 μ L) of eluent were used to measure inhibition of CaOx crystal growth via a ¹⁴C-oxalate incorporation assay, as reported elsewhere.¹⁷ Nephrocalcin isoforms were designated on the basis of the order of their elution at the following conductivity ranges: nephrocalcin A at 20 to 24 mS, nephrocalcin B at 25 to 32 mS, nephrocalcin C at 33 to 42 mS, and nephrocalcin D at 43 to 54 mS. Areas of each peak for the growth inhibitors of CaOx crystals (ie, nephrocalcin A, B, C, and D) were calculated, and the distribution of each isoform was expressed as a percentage of the total nephrocalcin concentration. The NaCl concentration was monitored via a conductivity meter^k calibrated by use of 0.1M KCl aqueous solution.

Urine creatinine concentrations were determined by use of an autoanalyzer.^l Urine pH and all buffer solutions were measured by use of a pH meter^m with a combination electrode. The pH meter was calibrated by use of standard buffers with a pH of 4 and 10 and standardized by use of buffer solutions with a pH of 5 and 7.

Statistical analysis—All data were normalized by dividing the concentration of each urinary analyte for a specific sample by the creatinine concentration for that sample. Study variables were reported as mean and SD. Differences between groups were assessed by use of a Student *t* test for paired samples. Statistical analysis was performed by use of statistical software.ⁿ Values of *P* < 0.05 were considered significant.

Results

Urine concentrations of total protein, glycosaminoglycans, and THP increased during consumption

Table 1—Mean \pm SD values for several urinary glycoprotein inhibitors of growth of CaOx crystals in urine obtained from 10 cats after they consumed a urolith prevention diet for 8 weeks or a regular diet being fed at the time of urolith detection.

| Variable | Regular diet | Urolith prevention diet | P value* |
|--------------------|---------------------|-------------------------|----------|
| Total protein | 0.552 \pm 0.467 | 0.805 \pm 0.467 | 0.281 |
| Glycosaminoglycans | 0.169 \pm 0.117 | 0.380 \pm 0.286 | 0.031 |
| THP | 0.489 \pm 0.580 | 0.604 \pm 0.481 | 0.529 |
| Nephrocalcin | | | |
| A + B (%) | 41.910 \pm 13.357 | 42.740 \pm 14.370 | 0.898 |
| C + D (%) | 58.090 \pm 13.357 | 59.260 \pm 14.345 | 0.868 |

Total protein, glycosaminoglycans, and THP concentrations were measured as mg/dL and divided by the urine concentration of creatinine (mg/dL) to normalize values for changes in urine volume. Nephrocalcin isoforms A, B, C, and D were each measured by calculating the area under the peak as they eluted from a DEAE cellulose column; values for the nephrocalcin isoforms are expressed as the percentage of the total nephrocalcin concentration.
*Values were considered significant at $P < 0.05$.

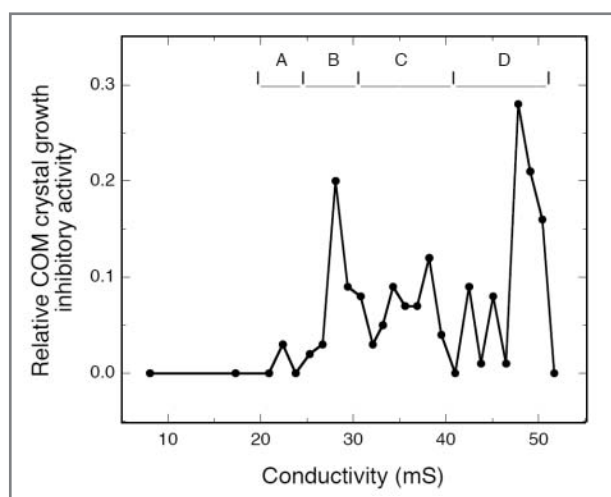


Figure 1—Elution pattern of nephrocalcin isoforms A, B, C, and D for DEAE cellulose column chromatography of a urine sample collected from a male cat with CaOx urolithiasis. The x-axis indicates NaCl concentration measured by a conductivity meter. The y-axis indicates the relative CaOx monohydrate (COM) crystal growth inhibitory activity in eluents separated by a DEAE cellulose column by use of ^{14}C -oxalate incorporation assay.

of the urolith prevention diet (Table 1). However, this increase was only significantly ($P = 0.031$) higher for glycosaminoglycans.

A typical nephrocalcin elution pattern of the urine collected from a male cat after it was fed the urolith preventive diet for 8 weeks was identified (Figure 1). The elution pattern was similar to that for human patients with CaOx uroliths.¹⁸ Distribution of the relative quantities of the 4 nephrocalcin isoforms was summarized (Figure 2). Diet did not affect relative urine concentration of individual isoforms. Similarly, diet did not affect relative urine concentration of isoforms when grouped as isoform A plus B and isoform C plus D (Table 1).

Discussion

In a previous study¹² conducted by our research group, consumption of the urolith prevention diet by urolith-forming cats reduced urinary CaOx supersaturation by 59%. This desired effect was likely a con-

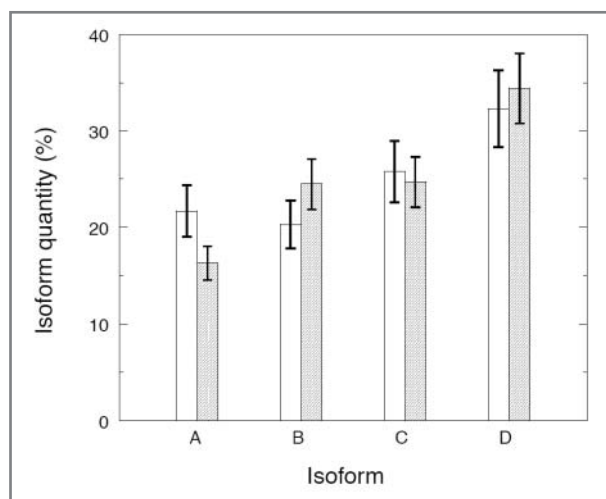


Figure 2—The effect of diet on the relative quantity of nephrocalcin isoforms in urine collected from 10 cats. Nephrocalcin isoforms were measured individually by calculating the area under the peak as they were eluted from a DEAE cellulose column. Values are expressed as a mean \pm SD percentage of the total nephrocalcin concentration. White bars indicate the isoform percentage when cats consumed their regular diet for 8 weeks; stippled bars indicate the isoform percentage when cats consumed a urolith prevention diet for 8 weeks.

sequence of the ability of the diet to reduce urinary concentrations of calcium, oxalate, and sodium and increase the urinary concentration of magnesium. Consumption of the diet was also associated with increased daily urine volume.

In the study reported here, it was found that in addition to reducing CaOx supersaturation, the urolith prevention diet also altered urinary concentrations of organic matrices that influence nucleation, growth, and aggregation of CaOx crystals. After consuming the urolith prevention diet for 8 weeks, urolith-forming cats produced urine with significantly higher concentrations of glycosaminoglycans, compared with the concentrations of glycosaminoglycans excreted during consumption of the diets fed prior to urolith detection. This finding is interesting because approximately half of human patients with CaOx uroliths have reduced concentrations of glycosaminoglycans, compared with concentrations in non-urolith formers.^{19–22} Therefore, increases in urinary concentrations of glycosaminoglycans may be an important consideration for prevention of the recurrence of CaOx uroliths.

Glycosaminoglycans are a heterogeneous group of compounds (eg, chondroitin sulfate, heparin sulfate, keratan sulfate, dermatan sulfate, and hyaluronic acid) that are naturally found in urine. Urine glycosaminoglycans are thought to originate from 2 sources (the metabolic turnover of connective tissue resulting in small fragments that enter the urine via glomerular filtration and larger fragments released from glomerular basement membranes and other urothelia). The manner by which diet influences urine concentration of glycosaminoglycans is unknown. Although supplement-type nutritional products containing glycosaminoglycans can be added to diets, the relative extent to which they are excreted in urine appears minimal. When pentosan polysulfate, a heterogeneous mixture of synthetic gly-

cosaminoglycans, was administered orally to rabbits, the percentage recovered in the urine was small and depended on the size of the molecule administered.²³ The median percentage of low-molecular-weight fractions of pentosan polysulfate recovered in urine was 7.4%; only 0.1% of high-molecular-weight fractions were recovered.

The mechanisms by which glycosaminoglycans disrupt urolith formation are not completely understood but may be related to their propensity to adhere reversibly to the surface of CaOx crystals.^{24–26} By decreasing the zeta potential of the crystal surface, glycosaminoglycans strengthen the electrical repulsive force between crystals, thereby inhibiting growth via crystal aggregation. This inhibitory activity is dependent on the degree of sulfation for a glycosaminoglycan and the distance between the sulfate ions in the molecule.²⁴ Furthermore, glycosaminoglycans have a strong anion potential. This negative surface charge may inhibit growth and nucleation of crystals through competition with negatively charged oxalate ions for the binding sites on positively charged calcium ions. In addition, glycosaminoglycans may protect the urinary mucosa by minimizing adherence of crystals.

In 1 study,²⁷ cats with urolithiasis had significantly higher concentrations of THP than did healthy cats without a history of uroliths. In that study,²⁷ the types of uroliths that cats formed were not provided. In the study reported here, feeding the urolith prevention diet to cats with CaOx urolithiasis had no effect on the magnitude of urinary concentrations of THP. However, the magnitude of THP concentrations may not be a reliable indicator of urolith inhibition. Urinary THP inhibits CaOx crystal aggregation in healthy humans.²⁸ However, THP isolated from human urolith formers is structurally and functionally different and accelerates the aggregation of CaOx crystals.²⁸ We did not investigate structural and functional differences of THP when cats consumed their regular diet versus the urolith prevention diet in the present study.

Nephrocalcin is an acidic urinary glycoprotein inhibitor of the growth of CaOx monohydrate crystals. Nephrocalcin has a molecular weight of approximately 14 kDa, and nephrocalcin from mammalian urine consists of at least 4 isoforms. Isoforms are designated as fractions A through D on the basis of the order of their elution during chromatography by use of a DEAE cellulose column with an NaCl gradient. Nephrocalcin from healthy humans without uroliths differs biochemically from nephrocalcin in urine of humans with CaOx uroliths. Isoforms A and B are strong inhibitors of growth of CaOx crystals and are relatively more abundant in urine of healthy humans. Humans with CaOx urolithiasis excrete nephrocalcin with decreased quantities of isoforms A and B and increased quantities of isoforms C and D, which are weak inhibitors of growth of CaOx crystals. Surface tension measurements by use of a Lauda film balance indicated that isoforms A and B are strongly amphiphilic and isoforms C and D are less amphiphilic.²⁹ The same results were detected with isoforms isolated from non-urolith-forming dogs (Beagles) and urolith-forming dogs (Miniature Schnauzers).³⁰ It is hypothesized that the hydrophilic or charged portion

of nephrocalcin becomes anchored to the surface of a CaOx crystal. Once attached, nephrocalcin forms a stable 2-D film that is able to cover the crystal surface, as determined by use of atomic force microscopy.³¹ With the hydrophilic portion attached to the crystal surface, the hydrophobic moieties are exposed to ions in the urine. The hydrophobic moieties facing the outside of the crystal reject attachment of ionic molecules. As a result, growth of CaOx crystals by attachment of new calculogenic ions is inhibited. These functional differences between nephrocalcin isoforms may be related to their composition of γ -carboxyglutamic acid residues. Only isoforms A and B contain γ -carboxyglutamic residues that change conformation on binding of calcium ions.³²

In the present study, the urolith prevention diet had no effect on the elution pattern of nephrocalcin isoforms isolated from the urine of cats with CaOx urolithiasis. This may suggest that the production and structure of nephrocalcin were controlled more by genetics than by dietary influences. Similar to results in other mammals with CaOx uroliths, the relative quantities of isoforms C and D were greater than the quantities of isoforms A and B in cats with CaOx. This finding suggests that nephrocalcin with reduced quantities of γ -carboxyglutamic acid residues may be a potential risk factor contributing to CaOx urolith formation in cats. The inability to detect a significant difference between dietary treatments may also have been attributable to the small number of cats in the study.

- a. Prescription Diet Feline c/d-oxl, Hill's Pet Nutrition Inc, Topeka, Kan.
- b. Iams Less Active Weight Control, Iams Co, Dayton, Ohio.
- c. Iams Original Formula, Iams Co, Dayton, Ohio.
- d. Meow Mix, Ralston Purina Co, St Louis, Mo.
- e. Moderate pH-0, Eukanuba Veterinary Diets, Iams Co, Dayton, Ohio.
- f. Science Diet Maintenance, Hill's Pet Nutrition Inc, Topeka, Kan.
- g. Senior, The Nutro Co, Franklin, Tenn.
- h. Whiskas Crave, Kal Kan Foods Inc, Vernon, Calif.
- i. Sigma-Aldrich, St Louis, Mo.
- j. Spectrophotometer DU 640, Beckman, Anaheim, Calif.
- k. CDM 2e, Radiometer Analytical, Copenhagen, Denmark.
- l. CX-5 autoanalyzer, Beckman, Anaheim, Calif.
- m. Ø71 pH meter, Beckman, Anaheim, Calif.
- n. SigmaStat, version 3.5, Systat Software Inc, Point Richmond, Calif.

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