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Defective Urinary Crystallization Inhibition and Urinary Stone Formation

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ABSTRACT

Introduction: Nephrocalcin (NC) is a glycoprotein produced in the kidney and inhibits calcium oxalate crystal formation. It has been separated into 4 isoforms (A, B, C, and D) and found that (A + B) are more abundant than (C + D) in urine of healthy subjects, but the reverse is seen in human urine of kidney stone patients. To further examine the role of this protein in inhibition of urinary crystallization, nephrocalcin isoforms were purified from 2 genetically pure dog species.
Materials and Methods: We studied healthy Beagles, known to be non-stone forming dogs, and Mini-Schnauzers, known to be calcium oxalate stone formers. NC was isolated and purified from each group. Urinary biochemistry and calcium oxalate crystal growth inhibition were measured.
Results: Specific crystal growth inhibition activity was significantly higher in non-stone forming dogs (9.79 ± 2.25 in Beagles vs. 2.75 ± 1.34 of Mini-Schnauzers, $p < 0.005$). Dissociation constants toward calcium oxalate monohydrate were 10-fold different, with Beagles' isoforms being 10 times stronger inhibitors compare to those of Mini-Schnauzers'. Isoforms C + D of NC were the main isoforms isolated in stone-forming dogs.
Conclusion: NC of these two species of dogs differently affects calcium oxalate crystallization and might have a role in determining ulterior urinary stone formation.

Key words*: kidney stone; calcium oxalate; crystallization; experiments; dogs*
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INTRODUCTION

Normal urine is supersaturated with respect to crystalline components, as a consequence of the essential homeostatic water conservation. This condition suggests the existence of physiological mechanisms that actively inhibit urinary crystallization of calcium salts (1). Various inhibitory macromolecules have been implicated in this process, e.g. osteopontin, crystal matrix protein, bikunin and nephrocalcin.

Nephrocalcin (NC) was isolated from human urine and kidney tissues (2), and later found in urine of 9 vertebrates' species (3). This glycoprotein is produced in proximal tubules in kidney (4) and its excretion is increased in renal carcinoma patients (5) and during pregnancy (6). NC has a mol. wt. of 14 kD, and can be separated into 4 isoforms with differ-

ent degrees of phosphorylation and amphiphilicities (7). Healthy subjects excrete more isoforms A and B that are less phosphorylated and have stronger hydrophobicity properties. In contrast, kidney stone forming patients excrete more isoforms -C and -D, which have higher degree of phosphorylation and weaker hydrophobicity. These isoforms of NC coat the surface of calcium oxalate crystals and control morphology, size and surface topography of crystals (8).

Evidence suggests that defective inhibitors can cause nephrolithiasis and NC accounts for a considerable portion of the inhibitory property of crystallization in urine (9). To examine this premise, we evaluated two species of pure-breed dogs with different incidences of kidney stones. We purified NC from urine samples from Beagles dogs, a non-stone forming species (10) and from Mini-Schnauzer dogs, known for frequent formation of calcium oxalate stones (11,12). In this report we compared chemical and physicochemical properties of NCs isolated from these species.

MATERIALS AND METHODS

Eleven healthy Beagles (3 neutered males and 8 neutered females, 4.0 ± 0.4 years old, body weight 9.28 ± 0.36 kg), and 7 Mini-Schnauzers (4 neutered males and 3 neutered females, 2.5 to 10.5 years old, body weight 6.6 ± 1.9 kg, who had at least one urinary stone) were selected. They were housed in individual cages under the conditions of controlled lighting and temperature, at the College of Veterinary Medicine, the University of Minnesota, according to the principles outlined in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

At the beginning of collection period, the urine from the bladders of dogs was emptied by transurethral catheterization. They were then housed in metabolic cages to facilitate complete collection of voided urine. Water was accessible throughout the collection period. Urine was collected in plastic containers surrounded by ice and stored in capped plastic containers with thymol at 4° C. To ensure complete removal of urine, dogs were catheterized at the end of 24 h. To minimize catheter induced bacterial urinary tract infection, cefadroxil® was administered orally (20 mg/kg, q 12 h) during the 24 h period of urine collection (13). Refrigerated urine samples were warmed at room temperature. Urine pH was measured by using a Beckman pHmeter. Calcium, citrate, creatinine, oxalate, phosphate, and uric acid were determined by using a Beckman CX-5 autoanalyzer. Protein was determined in urine by micro-Lowry method using Folin-Ciocalteu Phenol reagent (14). Bovine serum albumin was used as a calibration standard with a concentration range between 10 to 50 μg.

NC was isolated and purified by the method previously described (15). Briefly, urine was diluted 3-fold by distilled water, pH adjusted to 7.3, and added 1/10 volume of DEAE-cellulose pre-equilibrated in 0.05 M Tris-HCl, pH 7.3, then stirred gently for 30 min at room temperature. The DEAE cellulose was separated by filtering through Whatman #1 filter paper with a Buchner funnel. The DEAE-cellulose cake was then washed with 1 L of 0.05 M Tris-HCl, pH 7.3 containing 0.1 M NaCl (Buffer-A). NC was eluted by 200 mL of 0.05 M Tris-HCl, pH 7.3 containing 0.5 M NaCl (Buffer-B) with gentle stirring for 30 min at room temperature. The filtrated NC fraction was dialyzed against 10 L of distilled water overnight with 1 change. The dialyzed fraction was further subjected to a DEAE-cellulose column (2 x 15 cm), and 4 NC isoforms (A,B,C, and D) were isolated by a linear NaCl gradient using 125 mL each of Buffer-A and Buffer-B. The salt gradient was monitored by a conductivity meter (Radiometer CDM210). The quantity of the individual isoform inhibition as measured under the curve was calculated and expressed as a relative ratio of inhibitory activity by percentage. Each of the four NC isoforms was further purified by a molecular sieve column of BioRad P-10 column (2 x 85 cm) using 50% formamide for separating urobilirubin from nephrocalcin, then followed by Sephacryl S-200 (1 x 90 cm) using Buffer-A. Purified NC concentration was determined by an alkaline hydrolysis followed by a ninhydrin reaction (7). Phosphoric acid content was determined by the method described by Ames (16). The color was developed by using Fiske-Subbarow reagent and 0.01 M KH_2PO_4 was used for preparing a

calibration standard ranging between 50 to 500 μ moles of phosphate concentration.

Surface tension at the air-water interface was measured by Lauda film balance (Brinkman Instruments Co., Westbury, NY) using 0.01 M Tris-HCl, pH 7.4, containing 0.1 M NaCl, and applied 100 μg of protein over the surface of the buffer solution. The protein film was compressed from the surface area of 327 cm^2 to 18 cm^2 in 30 min and the pressure changes were monitored and recorded through a computer.

Calcium oxalate crystal growth inhibition was measured by either ^{14}C-calcium oxalate incorporation method (15) or spectrophotometric method measuring decrease of oxalic acid (2). In brief, ^{14}C-calcium oxalate incorporation assay was done by mixing 500 μL of sodium acetate buffer (50 mM acetic acid, 5 mM barbituric acid, 0.15 M NaCl, pH 5.7 containing 0.05 μCi/mL of ^{14}C-oxalic acid), 500 μL of calcium chloride solution (50 mM acetic acid, 2 mM $CaCl_2.2\ H_2O$, 0.13 M NaCl, 5 mM barbituric acid, pH 5.7) and 25 μL of a sample solution. The crystallization is initiated by adding 100 μL of calcium oxalate monohydrate crystal slurry (1.8 mg/mL in sodium acetate buffer, pH 5.7). After 40 minutes of incubation, the mixture was centrifuged, and radioactivity was measured in the supernatant. Inhibitory activity is calculated as the following equation: $I = (C_{40} - C_{blank})/(C_0 - C_{40}).(C_{blank})$ radioactivity of buffer solution; Co: radioactivity counts at initial time, C_{40}: radioactivity count in the supernatant after 40 min incubation. Spectrophotometric assay was performed as following: 1 mL of sodium oxalate was added to acetate buffer (8.75 mM acetic acid and 90 mM NaCl, pH 5.7) and to a calcium chloride solution in a cuvette, with an aliquot of the sample solution. While this mixture was stirring, 10 μL of calcium oxalate monohydrate slurry in acetate buffer (0.8 mg calcium oxalate monohydrate/mL of acetate buffer) was collected to the spectrophotometric analysis. As oxalic acid consumed to forming calcium oxalate, absorbance at 214 nm decreases. The slope of the curve reflects the strength of crystal growth inhibitory activity of a sample, and also the dissociation constant of an isolated inhibitor can be calculated by plotting a Langmuir isotherm type plot. Amino acid composition was determined by a Beckman amino acid analyzer (Model 119CL, Beckman Instruments, Palo Alto, CA), after hydrolysis in an evacuated tube containing 6 N HCl for 24 hrs at 11º C. Neutral sugar analysis was carried out by phenol-H_2SO_4 method (17). Calibration curve was made by using 5 to 20 μg of glucose aqueous solution. Molecular weight was determined by HPLC with a molecular sieve column (TSK-2000SW, ToSoHaas, Montogomeryville, PA). Molecular weight standards used were BSA, soybean trypsin inhibitor, and cytochrome C. The solvent used was a Buffer-B, and running conditions were isocratic mode, flow rate 1.0 mL/min, and detection wavelength at 220 nm.

Results are expressed as means ± SD. Statistical analyses were performed using Minitab 11.0 software. Group differences were compared by unpaired t-test and the frequency of nephrocalcin isoforms by χ^2-statistical analysis. A value of $p < 0.05$ was considered significant.

RESULTS

Urine of individual dogs was analyzed and averaged values of pH, calcium, phosphate, uric acid, citrate, oxalate, and protein are summarized in Table-1. There were significant differences in calcium and uric acid excretion between Mini-Schnauzers and Beagles ($p < 0.005$).

Urinary protein concentration and crystal growth inhibition activities of both species were compared in Table-2. The specific inhibitory activity of non-stone forming dogs (Beagles) was approximately

Table 1 – *Urine biochemistry. Concentrations are expressed as mg/creatinine basis.*

	Mini-Schnauzers (n = 7)	**Beagles** (n = 11)
pH	6.53 ± 0.72	6.79 ± 0.32
Protein	0.03 ± 0.01	0.02 ± 0.02
Calcium*	0.07 ± 0.02	0.03 ± 0.02
Uric acid*	0.17 ± 0.05	0.07 ± 0.02
Phosphate	0.52 ± 0.48	0.95 ± 0.38
Oxalate	0.04 ± 0.06	0.05 ± 0.01
Citrate	0.28 ± 0.40	0.12 ± 0.10

******* *Mini-Schnauzers vs. Beagles; $p < 0.005$.*

Table 2 – *Comparisons of urinary protein concentration and calcium oxalate crystal inhibition activity.*

	Protein Concentration (mg/mL)	**Specific Crystal Growth Inhibition Rate**
Beagles	0.21 ± 0.05	9.79 ± 2.25
Mini-Schnauzers*	0.23 ± 0.11	2.75 ± 1.34

**Inhibition rate, Beagles vs. Mini-Schnauzers; p < 0.005.*

4 times higher than stone forming dogs (Mini-Schnauzers).

Elution patterns of NC isoforms A,B,C, and D are summarized in Table-3. Non-stone forming Beagles excreted in their urine 56% of isoforms (A + B), and 44% of (C + D) isoforms. Particularly, B isoform peaked in the isoforms isolated. The ratios of isoforms in the Mini-Schnauzers dogs were 43% of isoforms (A + B) and 57% of isoforms (C + D). In this group, C was the main isoform isolated.

Carbohydrate content was measured by phenol-sulfonic acid method and calculated by molar ratio per protein (Table-4). All isoforms contained 0.01 to 0.02 g per gram of protein. Dogs urinary NC showed high content of acidic amino acid residues and low content of aromatic and basic amino acid residues. However, both species showed almost identical amino acid compositions. On the other hand, phosphate content in Mini-Schnauzers' NC was significantly higher compared to Beagles (Table-4).

Figure-1 shows typical force area curves of these isoforms at the air-water interface measured by a Lauda film balance. Isoform A of the Beagles showed the highest collapsing pressure, 44.8 mN/m, and gradual decreases were seen in B,C and D. Mini-Schnauzers showed lower collapse pressure in isoforms A and higher collapse pressure in isoforms C and D when compared to the Beagles' group. Table-4 summarizes collapsing pressure and dissociation constants of 4 NC isoforms isolated from both species.

Table 3 – *Distribution ratios (%) of 4 nephrocalcin isoforms.*

	Isoform-A	**Isoform-B**	**Isoform-C**	**Isoform-D**
Beagles (n = 11)*	11.45 ± 4.25	44.42 ± 14.31	21.05 ± 6.74	21.90 ± 13.37
Mini-Schnauzers (n = 7)	17.80 ± 6.41	26.79 ± 11.36	32.91 ± 14.13	18.19 ± 6.95

**Beagles vs. Mini-Schnauzers IsoA + IsoB, p < 0.05*

Table 4 – *Chemical composition and physical properties of 4 NC-Isoforms.*

	Beagles				**Mini-Schnauzers**			
Isoform	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Carbohydrate	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02
Phosphate	0.52	1.30	1.72	3.24	1.72	2.48	4.42	12.96*
Collapse pressure	44.8**	40.8**	27.4	31.0	35.8	31.2	33.7	36.9
Dissociation Constant**	$x10^{-7}$ M				$x10^{-6}$ M			

**Mini-Schnauzers vs. Beagles p < 0.005; ** Mini-Schnauzers vs. Beagles p < 0.05. Carbohydrate and phosphate are expressed in molar ratio to protein. Collapse pressure (Nm/m) was measured by a Lauda film balance. Dissociation constant toward calcium oxalate monohydrate crystals was determined by a spectrophotometric method and calculated from Langmuir isotherm type plot.*

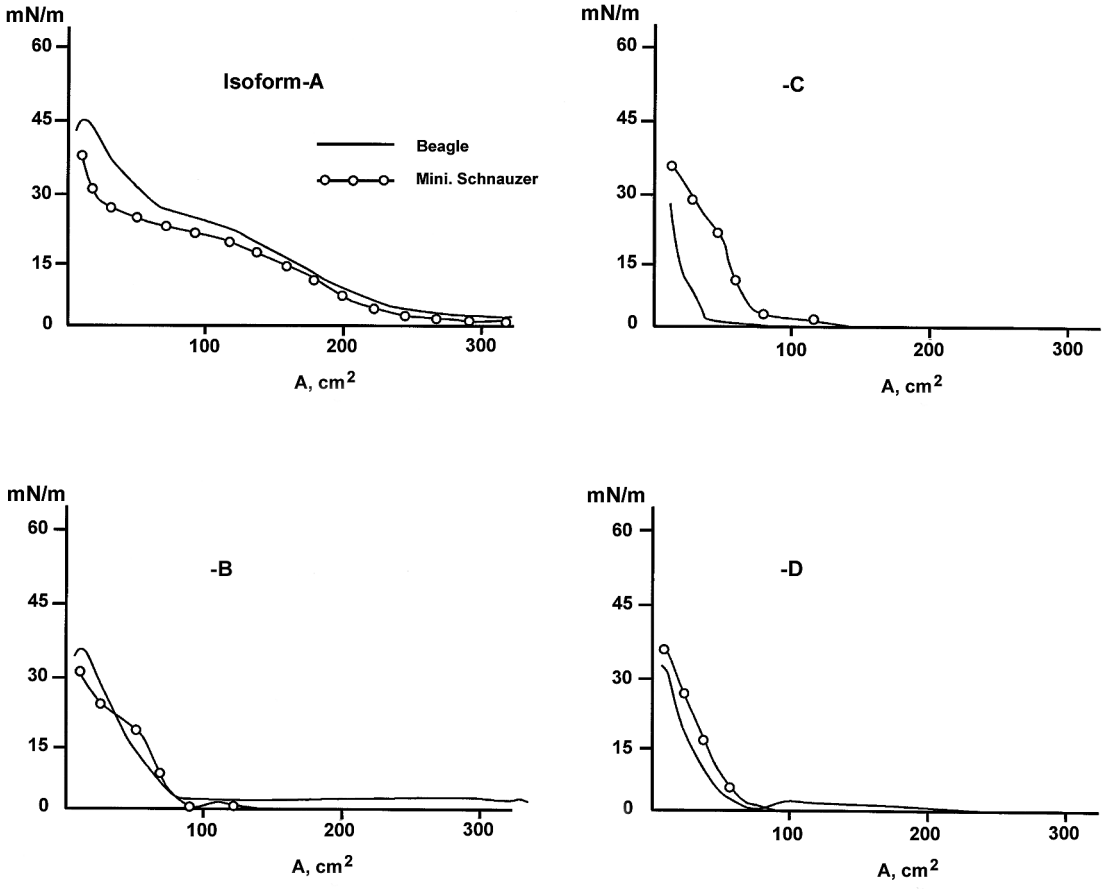

Figure 1 – *A force area curve of isolated isoforms at the air-water interface measured by Laud film balance. Experimental details are described in Materials and Methods section.*

COMMENTS

It has been accepted that stone formation is a crystallization process, taking place in supersaturated urine. However, despite nearly universal urine supersaturation, stones occur in a minority of people. Crystallization inhibitors, like citrate, proteins and glycosaminoglycans may account for this discrepancy (18). In this study, using two different species of dogs with distinct incidences of urinary stones several conclusions can be reached.

Mini-Schnauzers, a class of dogs with high formation of kidney stones, excreted more calcium and uric acid in their urine ($p < 0.005$) when compared to Beagle's group, a species that rarely presents with nephrolithiasis. These electrolytes combine to promote higher supersaturated urine, certainly predisposing these dogs to urinary stone formation.

However, we also found a remarkable difference in the qualitative excretion of urinary NC between these dogs' breeds. The NC isolated from the urine of Beagles showed strong inhibitory activity toward calcium oxalate crystals, but Mini-Schnauzers' urine has 4 times less inhibitory activity (Table-2). Previously we reported the same pattern of data in humans with or without urinary stones (7).

Non-stone forming Beagles' excreted in their urine more NC isoforms A and B than isoforms C and D when compared to Mini-Schnauzers ($p < 0.05$). NC isoforms are calcium-binding proteins, and binds

4 atoms of calcium ions per one molecule of NC. The Ca^{2+} binding mode is significantly different between isoform A or B and C or D: isoforms A-B binds Ca^{2+} directly through carboxyl groups of asp and/or glu residues. However, isoforms C-D requires at least two molecules of water between Ca^{2+} and carboxyl groups (19). Isoforms A and B changes its conformation and increase its hydrophobicity upon binding Ca^{2+}, but isoforms C or D do not. As a consequence isoforms C and D are more soluble in water and forms a less stable monolayer at the interface of air-water, and the collapsing pressure is lower. Less hydrophobicity of isoforms C and D might be related to a higher content of phosphate residues. In resume, we can say that in the current study we found the presence of a larger quantity of "good" inhibitors in Beagles' urine when compared to the group of Mini-Schnauzers dogs. Our classification of "good" inhibitors is based on different hydrophobicity, charge of the molecule and phosphate content. We can speculate at this moment that the isoforms that acts as strong (A-B) or weak (C-D) inhibitors of calcium oxalate crystallization are the same protein with different post-transcriptional modifications.

Finally, we found another indication of better inhibitory performance in the NC isolated from the urine of Beagles' group. When compared to the stone forming Mini-Schnauzer's dogs, dissociation constants toward CaOx monohydrate crystals for Beagles NC isoforms are 10-fold higher than those of stone forming dogs' isoforms. This means that they are at least 10-fold stronger in binding capacity to the CaOx crystal surface when compared to those of the Mini-Schnauzer NC.

We would like to point some limitations of our work. We did not study others inhibitors like glycosaminoglycans or Tamm-Horsfall protein, for example. However, we (20,21) have showed that almost 80% of inhibitory crystallization of calcium oxalate in urine is due to NC. Also, we made some inter-species analysis without using controls in each group. Nonetheless, this work can be considered as a pilot study and supported by these findings inter- and intra-species assessments will be the theme of a future analysis. Finally, our findings are not necessarily applicable to human nephrolithiasis. But in some cases animals' models of nephrolithiasis have close pathogenetic association to kidney stone formation in humans (22,23).

CONCLUSION

Nephrocalcin isolated from urine samples of Beagles dogs, a non-stone forming species, and from Mini-Schnauzer dogs, known for frequent formation of calcium oxalate stones, differently affected calcium oxalate crystallization and might have a role in determining urinary stone formation.

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EDITORIAL COMMENT

Nucleation, growth and aggregation are the principal crystallization mechanisms in stone development (1). Urine contains compounds that modulate such processes as well as the attachment of crystals to renal epithelial cells. While nucleation promotion activity is most likely sustained by membrane lipids, most of the inhibitory aggregation activity resides in macromolecules such as glycoproteins and glycosaminoglycans (2). Inhibitory proteins found in urine include nephrocalcin, Tamm-Horsfall glycoprotein (THG), prothrombin fragment 1, bikunin (uronic acid-rich protein), osteopontin, inter-α-trypsin inhibitor, among others. Most of the molecules are anionic, with many acidic amino acid residues, frequently contain post-translational modifications such as phosphorylation and glycosylation, and appear to exert their effects by binding to calcium oxalate surface (3). The specific structural motifs that favor crystal binding and inhibition are not yet known. A number of proteins are made by renal epithelial cells, whereas others gain access to the urine by glomerular filtration. In a number of cases, abnormalities of protein structure or function have been found in stone formers. It is not yet known what proportion of stone formers have an abnormality of inhibitor function (3).

Nephrocalcin, first described by Nakagawa et al. (4), has subsequently been shown in studies by the same group, to be deficient in gamma-carboxyglutamic acid in patients with calcium oxalate nephrolithiasis (5,6), hence reducing its ability to inhibit CaOx crystallization. According to Ryall (7), the fact that this protein has not been sequenced yet may raise some questions about it being possibly related to bikunin, a fragment of inter-α-trypsin inhibitor (8).

In this issue, Carvalho et al. compared biochemical and physicochemical properties of NC in 2 species of dogs that form (Mini-Schnauzers) or do not form (Beagles) calcium oxalate stones. They concluded that the specific crystal growth inhibition activity was significantly higher in the latter, which also possessed the isoforms usually not implicated in stone formation. Although these findings may not be applicable to human nephrolithiasis, it sheds further light into the understanding of how crystallization inhibitors may ultimately affect stone formation.

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