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Changes in cat urinary glycosaminoglycans with age and in feline urologic syndrome

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Abstract

The aim of the present study was to characterize the urinary excretion of glycosaminoglycans in kittens and adult healthy cats, as well as in cats with a low urinary tract disease, the feline urologic syndrome (FUS). The main urinary glycosaminoglycan in cats was found to be chondroitin sulfate, with smaller amounts of dermatan sulfate and heparan sulfate. There was no difference in the urinary glycosaminoglycan concentration with sex, but a marked decrease occurred with age, due to chondroitin sulfate. Trace amounts of keratan sulfate were also detected in the urine of kittens, but not of healthy adult cats. Dermatan sulfate and heparan sulfate were the only glycosaminoglycans found in the urinary tract and kidney, and chondroitin sulfate was the only glycosaminoglycan found in the plasma. These data suggest that the main urinary glycosaminoglycan chondroitin sulfate is of systemic origin and filtered in the kidney, while the minor components dermatan sulfate and heparan sulfate may come from the urinary tract. The urinary glycosaminoglycan concentration was greatly decreased in animals with FUS, as compared to normal adults. We hypothesize that these low glycosaminoglycan levels reflect a damage to the bladder surface, resulting in absorption and/or degradation of the endogenous urinary glycosaminoglycans.

Keywords: Cat; Glycosaminoglycan; Urine; Urinary tract; Kidney; Feline urologic syndrome

1. Introduction

Glycosaminoglycans are ubiquitous components of animal tissues, where they occur covalently linked to a protein core, forming proteoglycans (see review in Ref. [1]). Chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate and heparin are components of proteoglycans, and each has a unique tissue distribution pattern and structure [2,3]. Although all glycosaminoglycans are composed by disaccharide repeating units, there are considerable variations in their structure, achieved through biosynthetic modulation of sulfation, epimerization and acetylation reactions. For example, chondroitin sulfate and dermatan sulfate, the most common glycosaminoglycans of the extracellular matrix [4] and also referred to as "galactosaminoglycans" because they contain *N*-acetylgalactosamine as their hexosamine residues, are hybrid polymers. Chondroitin sulfate is composed of 4- and 6-sulfated disaccharide units, all of them containing D-glucuronic acid [5], and dermatan sulfate contains both D-glucuronic and L-iduronic acid residues [6]. The L-iduronic acid residues are introduced by a C-5 uronosyl epimerase [7], and differ from their D-glucuronic precursors by conformational flexibility; while D-glucuronic acid adopts a strict $^4C_1$ conformation, the L-iduronic acid residues oscillate freely between three different conformations, $^4C_1 - {}^2S_0 - {}^1C_4$ [8]. These structural characteristics are thought to confer the glycosaminoglycans and proteoglycans their functional properties.

The size of the proteoglycan core protein ranges from 10 to >500 kDa, and the number of glycosaminoglycan chains attached varies from 1 to >100. In addition, several proteoglycans carry glycosaminoglycan chains of more than one type (hybrid proteoglycans) and/or have additional *N*-linked or *O*-linked sugar modifications. Some proteins, such as MHC class II invariant chain and thrombomodulin, are "part-time" proteoglycans (see reviews in Refs. [4,9]).

The proteoglycan synthesis occurs in endoplasmic reticulum and Golgi. After synthesis, proteoglycans are transported from the Golgi to their destinations: the extracellular matrix, the cell surface or intracellular organelles. Such

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vectorial transport requires mechanisms for recognition, sorting and delivery [10]. Recognition and sorting must require determinants in the glycosaminoglycan chains and/or in the proteoglycan protein cores.

Proteoglycans are degraded by the sequential action of proteases, glycosidases, deacetylases and sulfatases, mostly in lysosomes. Most of the proteoglycan components is recycled, but small amounts of partially degraded glycosaminoglycans are excreted in the urine. Much of the information concerning the degradation of proteoglycans has been derived from the study of mucopolysaccharidoses, a group of genetic disorders characterized by accumulation in tissues and excretion in the urine of products that result from incomplete breakdown of proteoglycans, due to deficiency of one or more lysosomal hydrolases.

Human normal urine contains small amounts of glycosaminoglycans, and changes in urinary glycosaminoglycans have been recently reported in many diseases, such as renal lithiasis [11], interstitial cystitis [12], diabetes mellitus [13], and chronic renal failure [14]. Therefore, determination of glycosaminoglycans in the urine is gradually gaining importance in the literature. Nevertheless, little is known about urinary glycosaminoglycans in cats, and even less in cats with feline urologic syndrome (FUS), which is the most common disease of the low urinary tract in cats.

FUS is an idiopathic type of chronic cystitis that resembles, in many aspects, the human interstitial cystitis. The main clinical signs of this disease in cats are urinary frequency, urgency, hematuria and dysuria, without evidence of bacterial infection. Its etiology and pathogenesis are still undetermined, and its pathologic diagnosis is essentially one of exclusion, with no specific or clear criteria [15]. In male domestic cats, urethral obstruction by plugs composed of proteins, cellular debris and struvita crystals may lead to renal impairment and death.

Only one study on the urinary glycosaminoglycans in FUS has appeared [16]. The authors reported a decreased excretion of glycosaminoglycans in the urine, but individual glycosaminoglycans were not identified.

The objective of the present study was to identify and quantify the urinary glycosaminoglycans in kittens, in healthy adult cats and in FUS-affected cats, either with or without urethral obstruction. Urinary protein and creatinine were also quantified, and the glycosaminoglycans from plasma, kidney and urinary tract were characterized.

2. Materials and methods

2.1. Materials

Chondroitin 4-sulfate (from whale cartilage), chondroitin 6-sulfate (from shark cartilage), dermatan sulfate (from hog skin), papain, and Sigma Diagnostic Creatinine Kit

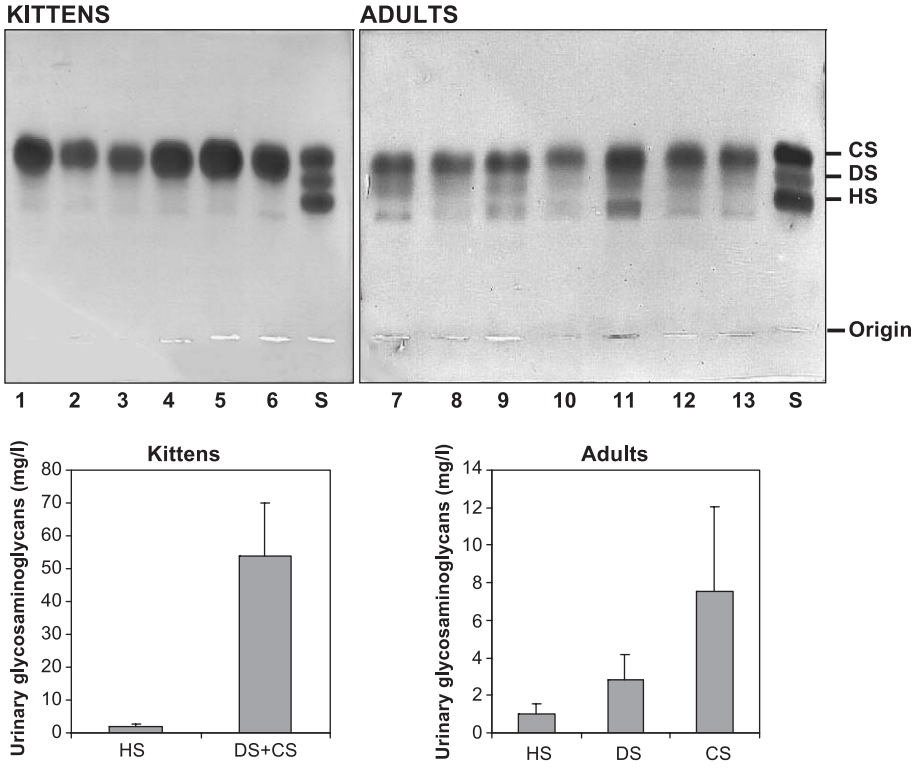

Fig. 1. Agarose gel electrophoresis of glycosaminoglycans from the urine of kittens (2–10 months old) and healthy adult cats (1–17 years old). The glycosaminoglycans were stained by toluidine blue, and quantified by densitometry. Graphs show mean + standard deviation for 32 kittens and 30 adult cats. S, mixture of standard glycosaminoglycans: chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS); 1–6, urinary glycosaminoglycans from six kittens; 7–13, urinary glycosaminoglycans from seven adult cats.

Table 1
Urinary glycosaminoglycans (GAGs) from kittens and normal adult cats

	GAG concentration (mg/l urine)				Creatinine (g/l urine)	GAG/creatinine ratio ($\times 10^{-3}$)			
	Chondroitin sulfate[a]	Dermatan sulfate[a]	Heparan sulfate	Total		Chondroitin sulfate[a]	Dermatan sulfate[a]	Heparan sulfate	Total
Kittens	53.99 ± 15.90 (97%)		1.92 ± 0.82 (3%)	55.91 ± 16.48 (100%)	2.81 ± 0.90	21.09 ± 8.09		0.73 ± 0.30	21.82 ± 8.30
Adult	7.54 ± 4.51 (66%)	2.82 ± 1.36 (24%)	0.99 ± 0.58 (8%)	11.89 ± 5.36 (100%)	4.25 ± 1.21	1.89 ± 1.33	0.70 ± 0.41	0.24 ± 0.15	2.73 ± 1.00

[a] The amounts of chondroitin sulfate excreted in the urine by kittens were so high that it was impossible to completely separate and quantify the dermatan sulfate band. These two galactosaminoglycans were quantified together for kittens.

(cat. no. 555-A) were purchased from Sigma Chemical Co., Inc. (St. Louis, MO, USA). Heparan sulfate (from bovine pancreas) [17], heparitinase II [18], and chondroitinases AC and B (from *Flavobacterium heparinum*) [19] were prepared by methods previously described. Agarose (standard, low M_r) and protein molecular size markers were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Acrylamide, *N,N*-methylenebisacrylamide, *N,N,N′,N′* -tetramethylethylenediamine, 1,3-diaminopropane and ethylenediamine were from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Disposable PD-10 desalting columns (Sephadex G-25 medium) and Q-Sepharose Fast Flow were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). MST1 anti-keratan sulfate monoclonal antibody was obtained as previously described [20]. Horseradish peroxidase rabbit anti-mouse IgG conjugate was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.2. Animals and urine samples

Urine samples were collected between 8:00 and 10:00 a.m. from 120 client-owned cats, which were divided in four groups: *Kittens*: 32 normal kittens (2.5–10 months old, 17 male and 15 female); *Normal*: 30 healthy adult cats (1–17 years old, 15 male and 15 female); *FUS*: 32 adult cats (1–14 years old, 26 male and 6 female) that presented to the Hospital of Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (HOVET-USP) with signs of FUS (hematuria and dysuria) without urethral obstruction; *FUS-O*: 26 male cats (1–7 years old) that presented to HOVET-USP with urethral obstruction caused by a plug. Urine samples (3–10 ml) were collected by either natural voiding or cystocentesis. The voided specimens were caught in mid-stream during micturition stimulated by manual compression of the bladder by means of palpation applied on abdomen just in front of the pelvic in let. The urine samples were centrifuged at 1000 × *g* for removal of debris, and the supernatants were stored at −20 °C until use. Creatinine, protein, and glycosaminoglycans were measured. Consent was given by the Ethical Committee of Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

2.3. Glycosaminoglycan identification and quantification

Glycosaminoglycans were identified by a combination of agarose gel electrophoresis and degradation with specific mucopolysaccharidases. Urine samples (1 ml) were desalted by gel exclusion chromatography on PD-10 desalting column (Sephadex G-25), freeze-dried in Speed Vac (VR-1 Heto Lab), and resuspended in 10 μl (for adult cats) or 20 μl (for kittens) of water.

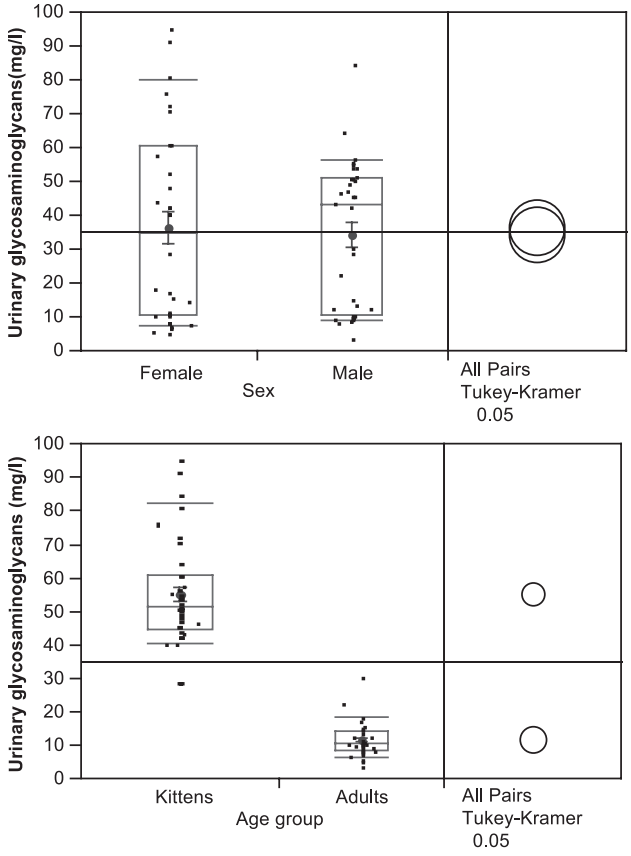

Fig. 2. Individual data for urinary glycosaminoglycan concentration of male and female, kittens and adult cats. Urinary glycosaminoglycans were quantified by densitometry of agarose gel slabs, as described in Fig. 1. The gray squares indicate median and 10% quantiles, horizontal lines indicate 25% quantiles, and gray dots with vertical lines indicate mean ± standard error. Circles refer to the Tukey–Kramer multiple comparisons test.

For large-scale preparation of urinary glycosaminoglycans, urine samples were pooled (90 ml for healthy adults and 103 ml for FUS-affected cats) and submitted to ion exchange chromatography on a Q-Sepharose column (2 × 3 cm, 10-ml bed volume). The column was eluted in a stepwise fashion with 0.3 M NaCl (30 ml), and then 2 M NaCl (30 ml). Three volumes of methanol was added to the fractions thus obtained, and the precipitate formed at − 20 °C overnight was collected by centrifugation and dried. The dried material was resuspended in water (1 ml). Only the 2 M fraction contained glycosaminoglycans.

Aliquots (5 μl) were submitted to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9, as previously described [21]. The glycosaminoglycans were quantified by densitometry (Scanner CS-9000, Shimadzu) of the gel slabs after fixation with cetyltrimethylammonium bromide and toluidine blue staining. These compounds were further identified by degradation with bacterial mucopolysaccharidases (chondroitinase AC, chondroitinase B and heparitinase II from *F. heparinum*), as previously described [22]. Briefly, aliquots of the glycosaminoglycans (50–100 μg) were incubated with $2–3 \times 10^{-4}$ units of either chondroitinase B, chondroitinase AC, heparitinase II, or a mixture of these enzymes, in 0.05 M ethylenediamine-acetate buffer, pH 8.0, in a final volume of 20 μl. After 6-h incubation at the optimum temperature of each enzyme, aliquots of the incubation mixtures were submitted to agarose gel electrophoresis, as described above. The degradation products do not precipitate with cetyltrimethylammonium bromide and disappear from the gel. To identify the degradation products, the incubation mixtures were spotted on Whatman No. 1 paper and chromatographed in isobutyric acid/1.25 M NH_4OH (5:3, v/v) for 24 h. The products formed were visualized by silver nitrate staining of the paper chromatograms and quantified by densitometry.

Polyacrylamide gel electrophoresis (PAGE) of the glycosaminoglycans was performed either in a BRL vertical mini-system [23] or in a horizontal system [24], after purification of each glycosaminoglycan by preparative agarose gel electrophoresis [6]. Gels were stained with toluidine blue.

Urinary keratan sulfate was detected by immunoblotting probed with MST1, a monoclonal antibody that recognizes both keratan sulfate free chains and keratan sulfate proteoglycans [20]. After agarose gel electrophoresis, glycosaminoglycans were transferred to nitrocellulose and Zeta-Probe membranes (Bio-Rad Laboratories). After blocking, membranes were probed with MST1. Then, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody. The antibody binding was visualized through diaminobenzidine (DAB), as previously described [25].

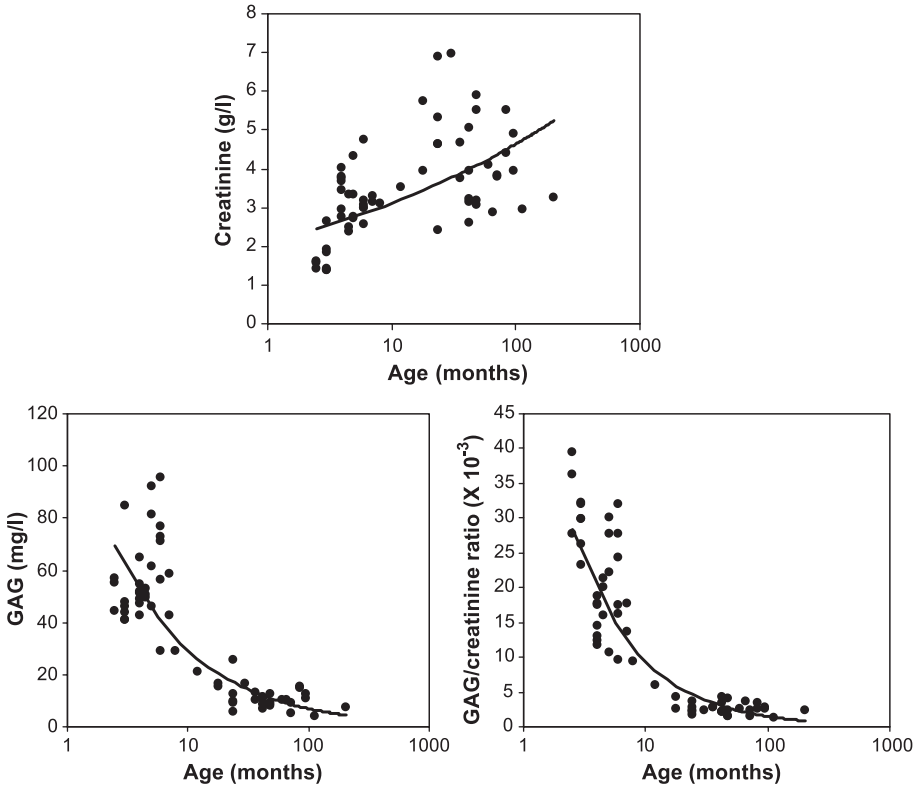

Fig. 3. Effect of animal age upon the urinary excretion of glycosaminoglycans (GAG) and creatinine. The experiment was performed as described in Fig. 1, except that the urinary creatinine and glycosaminoglycan (GAG) concentration, and the glycosaminoglycan/creatinine ratio were plotted as a function of the animal age, in months. Note that the age is expressed in logarithmic scale.

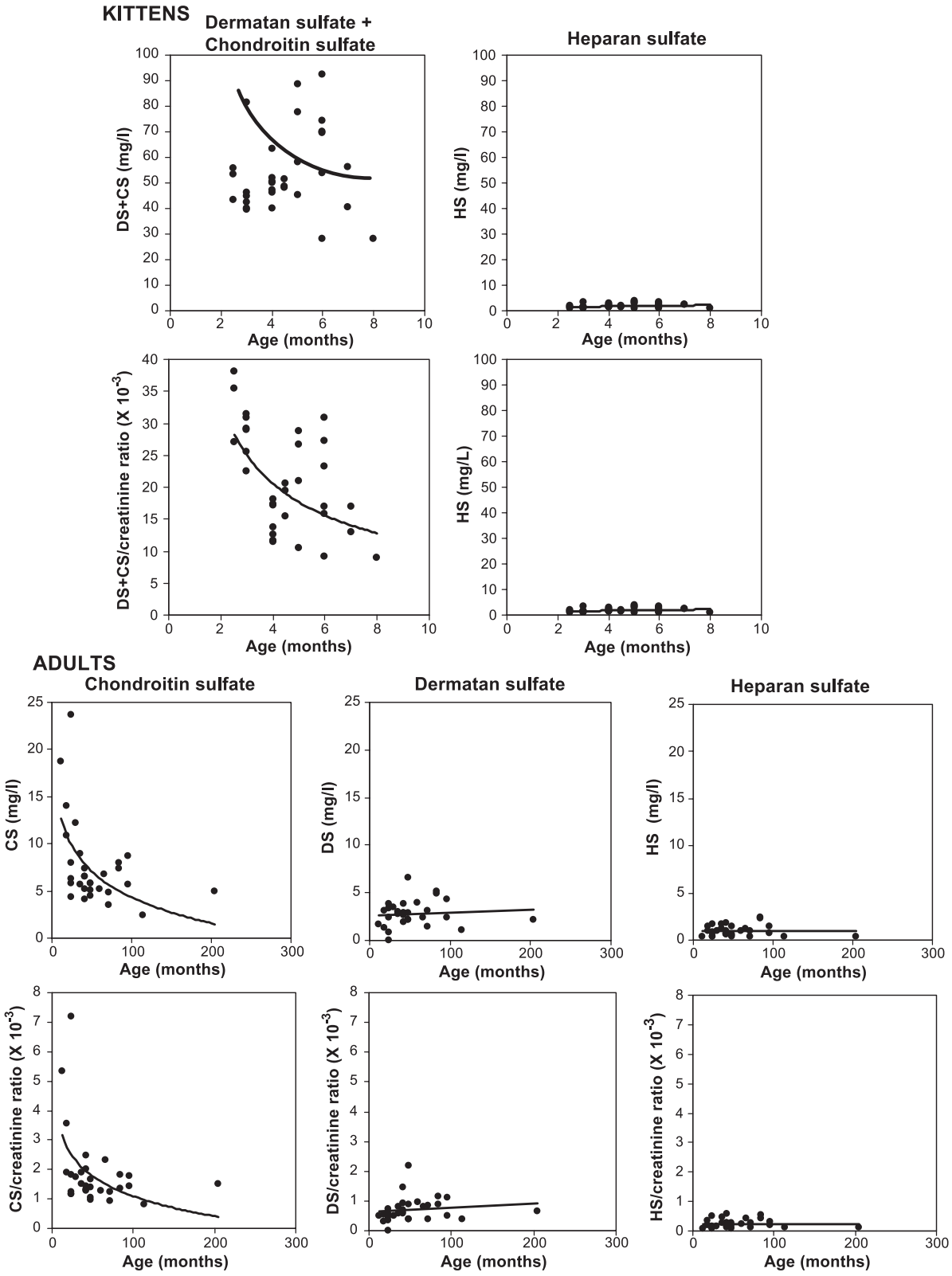

Fig. 4. Effect of cat age upon the urinary excretion of chondroitin sulfate, dermatan sulfate and heparan sulfate. The experiment was performed as described in Fig. 3, except that the urinary concentrations of chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) are shown for kittens and adult cats. Kittens excreted such large amounts of chondroitin sulfate in the urine that it was impossible to completely separate the dermatan sulfate band. So, these two galactosaminoglycans (dermatan sulfate and chondroitin sulfate, DS + CS) were quantified together for this age group.

2.4. Isolation, identification, and quantification of tissue and plasma glycosaminoglycans

Kidneys, ureters, bladder, urethra, and penis were obtained on necropsy from adult cats, at HOVET-USP. Only animals that did not present any signals of urologic disease were selected. The tissues were weighed, ground in 10 volumes of acetone and, after standing overnight at room temperature, the fragments were collected by centrifugation and vacuum-dried. The dried material was resuspended in 0.08 M phosphate-cysteine buffer pH 6.5, containing 0.02 M EDTA and 100 mg/l papain (10 ml/g of dry material). After overnight incubation at 50 °C, the samples were cooled in an ice bath, and trichloroacetic acid and NaCl were added to final concentrations of 5% and 1 M, respectively. After standing 15 min in the ice bath, the precipitate formed was removed, the pH of the supernatant was adjusted to 7.0, and 2 volumes of ethanol was added. The precipitate formed after 24 h at −20 °C was collected by centrifugation and dried. The dried material was resuspended in water and the glycosaminoglycans were identified by agarose gel electrophoresis and enzymatic degradation with mucopolysaccharidases, as described above.

Blood samples (2 ml) from healthy adult cats were collected in the presence of EDTA, and plasma was obtained after centrifugation. Each sample was held in alkaline conditions (0.5 M NaCl) at 37 °C for 12 h to cleave covalent *O*-linkages between protein and carbohydrate and to release the glycosaminoglycan chains from proteoglycans or peptidoglycans. The glycosaminoglycan chains were isolated by ion exchange chromatography of the neutralized samples on Q-Sepharose Fast Flow (0.7 × 3-cm column) in chloride form. The column was washed with 10 ml of 0.3 M NaCl, and the glycosaminoglycans were eluted by 1 M NaCl (five 1 ml fractions). Three volumes of methanol was added to the fractions, and after standing overnight at −20 °C, the precipitate formed was collected by centrifugation, vaccum-dried, resuspended in 10–20 μl of water, and analyzed by agarose gel electrophoresis, as described above.

2.5. Quantification of protein and creatinine

Protein was measured by the Coomassie blue Method according to Spector [26], adapted to micro-scale: aliquots (10 μl) of urine and 200 μl of the Coomassie blue reagent were added to a 96-well plate. The absorbance was read at

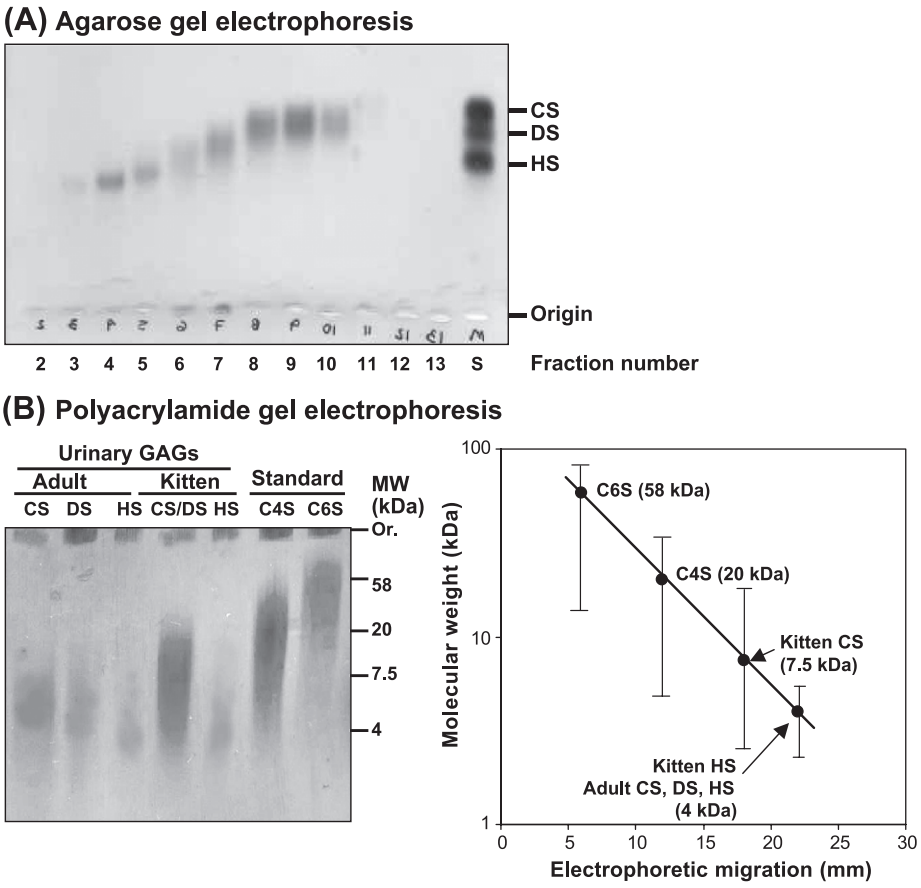

Fig. 5. Agarose gel electrophoresis of fractions obtained by preparative agarose gel electrophoresis of urinary glycosaminoglycans (A), and PAGE of these compounds (B). The experiment was performed as described in the text, and the distance of migration versus modal molecular weight was plotted. All urinary glycosaminoglycans were polydisperse, and the vertical lines in the graph indicate the range of molecular weights. The abbreviations used are: CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; C6S, chondroitin 6-sulfate; C4S, chondroitin 4-sulfate; S, mixture of standard glycosaminoglycans.

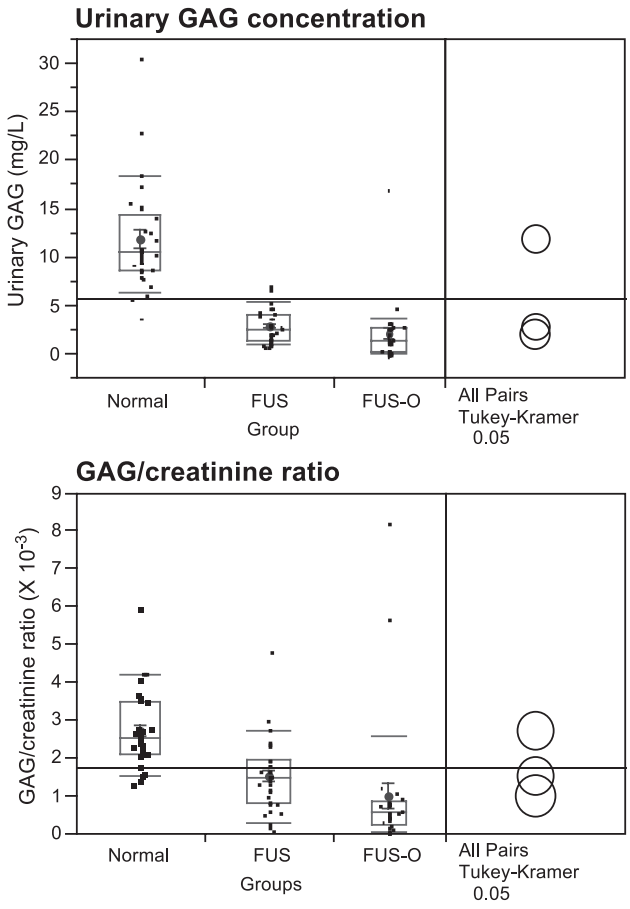

Fig. 6. Urinary glycosaminoglycan concentration and glycosaminoglycan/creatinine ratio for normal adult cats and for cats with FUS, without or with urethral obstruction (FUS-O). Urinary glycosaminoglycans (GAG) were identified and quantified as described in Fig. 1. The gray squares indicate median and 10% quantiles, horizontal lines indicate 25% quantiles, and gray dots with vertical lines indicate mean ± standard error. Circles refer to the Tukey–Kramer multiple comparisons test.

630 nm in a Microplate Reader (ELISA ELX-800 Universal, BioTek Instruments, Inc.). The urinary proteins were also submitted to agarose gel electrophoresis, fixed by formol/methanol (1:4, v/v), and stained by Amido Black (0.1% in 50% ethanol/1% acetic acid).

Creatinine was measured by the alkaline picrate method, using the Sigma Diagnostics Kit, also adapted to microscale: urine samples were diluted (1:10) and 5-μl aliquots were mixed with 15 μl of water and 200 μl of the alkaline picrate solution in a 96-well plate. The absorbance was read at 490 nm in the Microplate Reader.

2.6. Statistical analysis

The test of Anderson–Darling revealed that the sample distributions were nonparametric. The Kruskal–Wallis nonparametric sample rank test for independent groups was used to compare the mean difference in urinary glycosaminoglycans, protein and creatinine from normal adult cats, kittens and cats with FUS, with or without urethral obstruction. When the differences between the affected and normal groups were found to be significant, the Tukey test for multiple comparisons was also applied.

3. Results

Fig. 1 shows representative agarose gel slabs of glycosaminoglycans extracted from the urine of healthy kittens and adult cats. The main band migrates as chondroitin sulfate for both age groups, but kittens excreted very large amounts of this glycosaminoglycan, with smaller amounts of dermatan sulfate and heparan sulfate. To confirm the glycosaminoglycan identification, these compounds were submitted to the action of specific mucopolysaccharidases. The band migrating as chondroitin sulfate was totally degraded by chondroitinase AC, whereas the band migrating as dermatan sulfate was susceptible to the action of chondroitinase B. These glycosaminoglycans were resistant to heparitinase II, which degraded the band that migrated as heparan sulfate. These compounds were quantified by densitometry of the agarose gel slabs. Fig. 1 also shows the results obtained (mean ± standard deviation). The amounts of chondroitin sulfate excreted by kittens were so high that it was impossible to separate the dermatan sulfate. So, these two galactosaminoglycans (chondroitin sulfate and dermatan sulfate) were quantified together.

Table 1 shows the quantitative results, expressed both as concentration of urinary glycosaminoglycans (mg/l) and as glycosaminoglycan/creatinine ratio. Kittens (2.5–10 months old) excreted much more glycosaminoglycans in the urine than adults (1–17 years old). No statistically significant differences were observed between male and female cats of the same age group (Fig. 2), but kittens excreted much more glycosaminoglycans in the urine than adults ($P < 0.05$).

Table 2
Urinary glycosaminoglycans, creatinine and protein in cats with feline urological syndrome, either with (FUS-O) or without (FUS) urethral obstruction

	Urinary glycosaminoglycans (mg/l)				Protein (g/l)	Creatinine (g/l)	GAG/creatinine ($\times 10^{-3}$)
	Chondroitin sulfate	Dermatan sulfate	Heparan sulfate	Total			
Normal	7.54 ± 4.51 (65%)	2.82 ± 1.36 (25%)	0.99 ± 0.58 (9%)	11.89 ± 5.36 (100%)	0.25 ± 0.09	4.21 ± 1.22	2.97 ± 1.60
FUS	3.68 ± 3.73* (70%)	1.24 ± 1.22* (21%)	0.52 ± 0.91* (10%)	5.79 ± 5.26* (100%)	0.60 ± 0.36*	3.64 ± 1.86	1.53 ± 0.98*
FUS-O	1.26 ± 1.01* (78%)	0.81 ± 2.78* (15%)	0.09 ± 0.11* (7%)	2.01 ± 2.26* (100%)	0.80 ± 0.27*	2.79 ± 1.67*	1.04 ± 1.81*

* Difference statistically significant from control, $P < 0.001$.

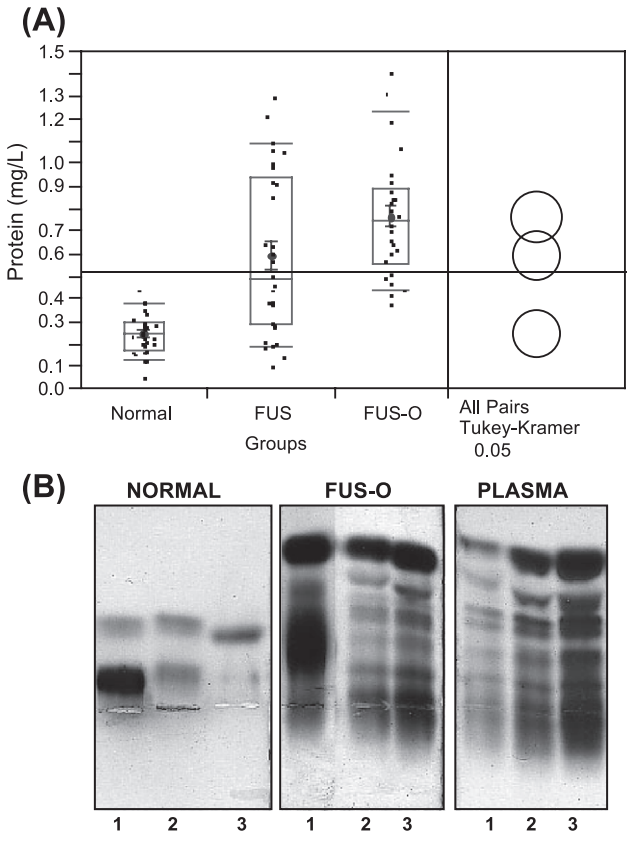

Fig. 7. (A) Urinary protein concentration in normal cats and in cats with FUS, without or with urethral obstruction (FUS-O), and (B) agarose gel electrophoresis of urinary proteins from Normal and FUS-O cats, and plasma proteins. The experiment was performed as described in Section 2. 1–3, urine and plasma samples obtained from three normal cats and from three cats with FUS and urethral obstruction.

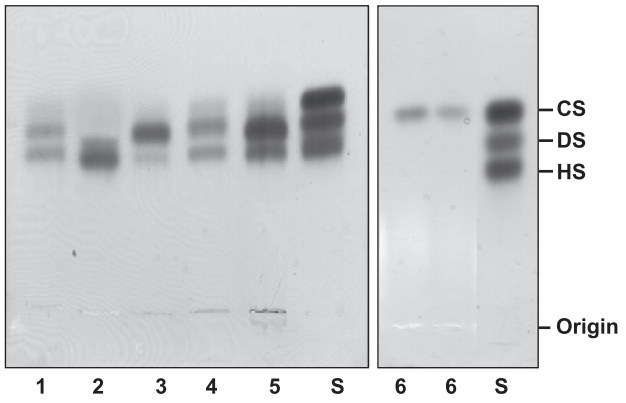

Fig. 9. Agarose gel electrophoresis of glycosaminoglycans extracted from kidney and urinary tract of adult cat. Glycosaminoglycans were extracted from cat penis (1), kidney (2), urethra (3), ureter (4), bladder (5), and plasma (6) as described in the text. Aliquots were submitted to agarose gel electrophoresis. S, mixture of standard glycosaminoglycans: chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS).

Fig. 3 shows that both the glycosaminoglycan concentration and the glycosaminoglycan/creatinine ratio decreased logarithmically with age. In contrast, the urinary creatinine increased with age, as expected. The decrease in urinary glycosaminoglycan was mainly due to chondroitin sulfate (Fig. 4). In adults, only chondroitin sulfate decreased with age—the concentrations of dermatan sulfate and heparan sulfate remained constant. In kittens, the concentration of galactosaminoglycans (chondroitin sulfate and dermatan sulfate, quantified together) also decreased with age.

To determine the molecular weight of the glycosaminoglycans excreted in the urine by kittens and adult cats, these compounds were fractionated by preparative-scale agarose gel electrophoresis (Fig. 5A), and the fractions thus obtained, corresponding to chondroitin sulfate, dermatan sulfate and heparan sulfate, were submitted to PAGE (Fig. 5B). All the urinary glycosaminoglycans were polydisperse and of low molecular weight.

Concerning the urinary glycosaminoglycan excretion in cats with FUS, a statistically significant decrease was ob-

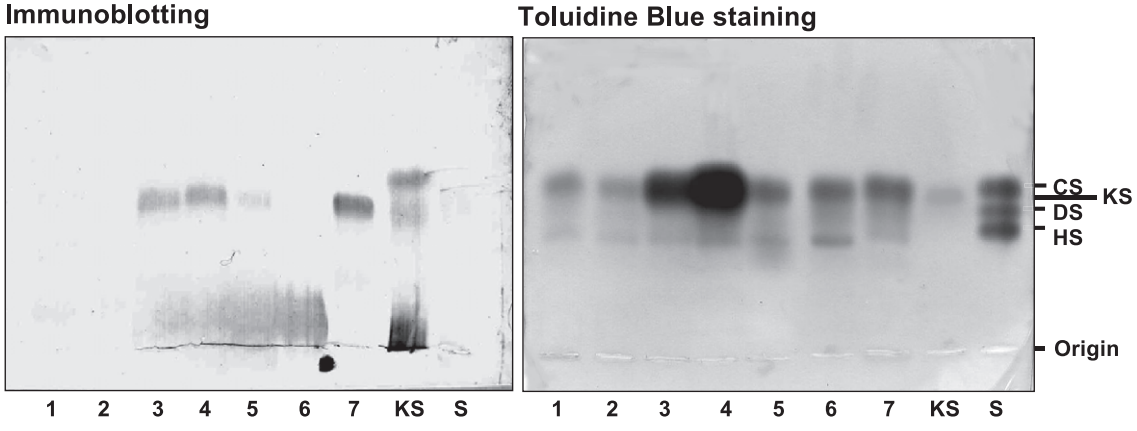

Fig. 8. Immunoblotting after agarose gel electrophoresis of glycosaminoglycans extracted from the urine of healthy kittens and adult cats and of cats with FUS. The experiment was performed as described in Fig. 1 except that, after the agarose gel electrophoresis, the products were transferred to nitrocellulose membrane and stained as described in Methods. KS, keratan sulfate; S, mixture of standard glycosaminoglycans: chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS); 1, 2, 6, urinary glycosaminoglycans from adult normal cats; 3–4, urinary glycosaminoglycans from kittens; 5, 7, urinary glycosaminoglycans from adult cats with FUS.

Table 3
Glycosaminoglycans (GAGs) of the kidney, urinary tract, and plasma of normal adult cats

Tissue	Total GAG (μg/g dry tissue weight or μg/ml plasma)	Chondroitin sulfate (μg/g dry tissue weight or μg/ml plasma)	Dermatan sulfate (μg/g dry tissue weight or μg/ml plasma)	Heparan sulfate (μg/g dry tissue weight or μg/ml plasma)
Kidney	66.65 (100%)	n.d.	16.00 (24%)	50.66 (76%)
Ureters	59.12 (100%)	n.d.	42.39 (72%)	16.73 (28%)
Bladder	210.08 (100%)	n.d.	133.38 (63%)	76.69 (37%)
Urethra	232.29 (100%)	n.d.	195.65 (84%)	36.64 (16%)
Penis	82.47 (100%)	n.d.	53.55 (65%)	28.93 (35%)
Plasma	4.08 ± 0.24[a] (100%)	4.08 ± 0.24[a] (100%)	n.d.	n.d.

n.d. = not detected.
[a] Mean ± standard error of five samples.

served, in comparison to age-matched healthy controls (Fig. 6). This decrease was observed in all animals with FUS, regardless of urethral obstruction (FUS and FUS-O groups). These animals also excreted increased amounts of protein, and decreased amounts of creatinine in the urine, as compared to the normal group. Nevertheless, the glycosaminoglycan excretion dropped even more than the creatinine, since the glycosaminoglycan/creatinine ratio also was lower than normal (Table 2). Besides, the relative amounts of chondroitin sulfate were above normal (statistically significant only for the FUS-O group). Fig. 7 shows that the animals with FUS presented proteinuria as compared to normal cats (difference statistically significant, $P<0.05$) and that the electrophoretic migration of the urinary proteins in FUS-O group is similar to the electrophoretic pattern of plasma proteins, suggesting that the increase in urinary protein is due to hematuria. Small amounts of keratan sulfate were also detected, by immunoblotting, in the urine of kittens and in the urine of cats with FUS, but not of healthy adult cats (Fig. 8).

In contrast to the urine, Fig. 9 and Table 3 show that the main glycosaminoglycans found in the cat kidney and urinary tract were heparan sulfate and dermatan sulfate, respectively. Only trace amounts of chondroitin sulfate were detected in some of these tissues. In contrast, chondroitin sulfate was the only glycosaminoglycan detected in the plasma of these animals.

4. Discussion

The exact mechanism behind the urinary excretion of glycosaminoglycans is not completely understood. We have previously shown that chondroitin sulfate administered to rats (i.p. injection) is excreted in the urine, part as polymeric chondroitin sulfate and part as low molecular weight degradation products [27]. Hurst [28] has shown that cystectomy in rats did not produce detectable changes in excreted glycosaminoglycans. These results indicate that most of the urinary glycosaminoglycans originate in the kidney, and not from the bladder.

Different methods have been used to measure and identify the urinary glycosaminoglycans. Reliable results are usually obtained by methods based on precipitation with either cetylpyridinium chloride [29] or cetyltrimethylammonium bromide [30], ion exchange chromatography [31], or desalting and concentration [32,33]. Direct measurements of glycosaminoglycans in urine samples by colorimetric methods, such as complexation with Alcian blue [34], spectrophotometry with 1,9-dimethylmethylene blue (DMB) [35], or determination of uronic acid [36] are unreliable, since other urinary components, such as salts and nucleic acids, may interfere, leading to false results. The identification of different glycosaminoglycans is usually achieved by either cellulose acetate [33] or agarose gel [21] electrophoresis, associated with differential degradation with specific mucopolisaccharidases [11,14,22] and/or nitrous acid [31]. In 1989, we compared different methods for isolation and identification of human urine glycosaminoglycans, and concluded that the best results were obtained when urine samples were desalted, concentrated and the glycosaminoglycans were quantified by densitometry after agarose gel electrophoresis. The identification of each glycosaminoglycan was confirmed by degradation with specific mucopolysaccharidases [11]. In the present study, we used both the desalting-concentration method and an ion exchange chromatography-based procedure, followed by a combination of agarose gel electrophoresis and degradation with *F. heparinum* chondroitinase B, chondroitinase AC and heparitinase II.

Concerning the cat, only few reports on the urinary excretion of glycosaminoglycans have been published, most of them describing mucopolysaccharidosis-affected animals [37,38,39,40]. Buffington et al. [16] reported a decreased excretion of glycosaminoglycans in the urine of cats with interstitial cystitis, using the spectrophotometric DMB method, but individual glycosaminoglycans were not identified.

In the present paper, we described the urinary excretion of glycosaminoglycans in healthy cats of different ages, and in cats with FUS.

The main urinary glycosaminoglycan in cats was found to be chondroitin sulfate, with smaller amounts of dermatan sulfate and heparan sulfate. Kittens excreted much more glycosaminoglycans in the urine than adults, both expressed as glycosaminoglycan concentration (mg/l) and as glycosaminoglycan/creatinine ratio. This finding agrees with data previously reported [30]. The creatinine concentration increased with age as expected, since it is proportional to the muscular mass of the animal (2.4 g/l for 2.5–4-month-old cats, and 4.2 mg/l for cats older than 30 months), but the glycosaminoglycan concentration decreased much more

(51.4 mg/l for 2.5–4-month-old cats, and 10.3 mg/l for cats older than 30 months). We observed that this marked decrease in urinary glycosaminoglycans is mainly due to chondroitin sulfate. The chondroitin sulfate decreases even in adult cats (older than 12 months). Furthermore, kittens excreted trace amounts of keratan sulfate in the urine, detectable by immunoblotting, in contrast to healthy adults that did not. All urinary glycosaminoglycans were of low molecular weight, indicating that they are degradation products of high molecular weight proteoglycans.

Furthermore, we have also shown that chondroitin sulfate is not present in the cat kidney and urinary tract. The only glycosaminoglycans found in the kidney and urinary tract were dermatan sulfate and heparan sulfate. In contrast, chondroitin sulfate was found in the cat plasma. These data suggest that the urinary chondroitin sulfate is of systemic origin, possibly filtered in the kidney. The urinary keratan sulfate, a minor component, possibly comes also from cartilage. On the contrary, the urinary heparan sulfate and dermatan sulfate may be either originated from the urinary tract and kidney or may be filtered.

Circulating glycosaminoglycans are supposed to represent products of the metabolism of connective tissues, en route to catabolism in liver or excretion in the urine. Cartilage, muscle, connective tissue, vascular endothelial cells and blood cells are among the possible sites of origin of plasma and serum glycosaminoglycans. The high concentration of chondroitin sulfate in the urine of kittens is possibly due to the high metabolic, tissue remodeling, and glycosaminoglycan turnover rates that occur in these animals.

All urinary glycosaminoglycans were greatly decreased in animals with FUS, with or without urethral obstruction. When urethral obstruction was present, the differences were even higher in comparison to normal animals. Nevertheless, this decrease was smaller for chondroitin sulfate than for the other glycosaminoglycans.

Urinary creatinine concentration was decreased in animals with urethral obstruction, possibly due to acute renal failure. These animals also presented proteinuria, due to hematuria, and the presence of keratan sulfate in the urine of these animals possibly reflects the hematuria.

FUS closely resembles human interstital cystitis. Because interstitial cystitis (and FUS) involves a chronic non-bacterial inflammation of the bladder wall, one of the most popular concepts has been that the urothelium is defective, allowing noxiuos substances to diffuse into the bladder wall, causing the symptoms [41]. Since glycosaminoglycans were thought to protect bladder urothelium from bacterial and microcrystal adherence, it had been postulated that interstitial cystitis patients might have a defective glycosaminoglycan layer [42]. Nevertheless, our results and recently published data by other authors indicate that it is not so.

Stains such as periodate-Schiff, Alcian blue, ruthenium red, and colloidal iron strongly stain the surface of animal bladders, suggesting the presence of a layer of acidic glycoconjugates [43,44]. Observations such as loss of surface material after mild acid or protamine treatment led investigators to suggest the presence of a coat of glycosaminoglycans on the bladder surface [45]. However, the precise nature and biochemical composition of glycoconjugates cannot be deduced from histochemical staining. Moreover, under standard conditions, glycosaminoglycans are not sufficiently oxidized by periodate to yield aldehyde groups for reaction with Schiff reagent. Therefore, urothelium has to have considerable concentration of other glycoconjugates to account for the observed periodate-Schiff staining. In fact, subsequent studies of the bladder have clearly shown that glycoproteins rather than glycosaminoglycans are the major components of the bladder epithelium, and that the former include a mucin [46,47].

On the basis of the data here presented, we hypothesize that the low glycosaminoglycan levels in cats with FUS reflect a damage to the bladder surface, resulting in absorption of the endogenous, kidney-filtered, urinary glycosaminoglycans. Another possibility is that these animals might have a decreased concentration of circulating glycosaminoglycans. Future studies to compare plasma glycosaminoglycans in healthy cats to cats with FUS would help to elucidate this point, and also the mechanism of action of treatments in current clinical use with pentosanlpolysulfate, heparin or hyaluronan, presumably to ‘‘replace’’ glycosaminoglycans in interstitial cystitis and FUS.

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