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Assessment of urinary inhibitor or promoter activity in uric acid nephrolithiasis

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Abstract

Purpose—To assess the presence of a reduced inhibitor activity or an increased promoter activity in urine of idiopathic uric acid stone formers (IUASF) compared to non-stone formers (NSF) independent of urinary pH.

Methods—30 IUASF, 9 obese NSF and 12 lean NSF collected 24-hour urine under metabolic diet. Three urine aliquots per subject were used to assess spontaneous nucleation (SN, *de novo*

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CONFLICT OF INTEREST

None.

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crystal formation), crystal growth (CG) using a 0.1 mg/mL seed of anhydrous uric acid (UA) and steady state (SS) of UA solubility using a 5 mg/mL seed of UA (assessing maximum amount of UA dissolvable in urine). All experiments were conducted for 6 hours at a constant pH of 5.0. UA concentration was measured in filtered aliquots at 0, 3 and 6 hours.

Results—At baseline, 24-hour urinary pH was significantly lower and UA saturation significantly higher in IUASF. No significant SN occurred and a similar SS UA concentration was reached in the three groups. IUASF and lean NSF displayed a similar decrease in UA concentration during CG, while obese NSF started with higher UA concentration and consequently displayed higher magnitude of decrease in UA concentration for CG.

Conclusions—This study suggests that there is no significant difference between IUASF and NSF in terms of promoter or inhibitor activity in whole urine against UA stone formation when urine pH is maintained constant. The findings suggest that UA stone formation is dictated by a high urinary saturation with respect to UA, driven primarily by a low urine pH.

Keywords

kidney stones; urolithiasis; uric acid; crystallization; inhibitor

INTRODUCTION

Uric acid (UA) stones account for 8–10% of all kidney stones.¹ Low urinary pH is the major factor influencing UA solubility which decreases drastically when pH is persistently below 5.5 (pK_a for UA), leading to increased urinary content of undissociated UA and its subsequent precipitation.^{1–4} In addition to idiopathic uric acid stone formers (IUASF), low urinary pH is associated with obesity, type 2 diabetes (T2D) and metabolic syndrome, all conditions in which UA stones is more prevalent.^{4–13} Patients with T2D with no kidney stones exhibit low urinary pH of similar magnitude to that of IUASF.^{4,14,15} However, only a fraction of obese individuals and T2D develop UA stones, suggesting that a low urinary pH is necessary but not sufficient for UA stone formation. One hypothesis is that IUASF may have additional urinary features such as increased promoter or impaired inhibitor activity against UA stone formation. This hypothesis is supported by studies in calcium oxalate (CaOx) stone formers in whom stone formation involves a breakdown of the normal balance between CaOx supersaturation and urinary promoters and inhibitors of CaOx crystal nucleation and growth.¹⁶ Some studies have assessed the role of specific inhibitors on UA crystallization using synthetic urine,¹⁷ but none have evaluated for urinary promoter and inhibitor activity against UA crystal nucleation and growth in whole urine.

Our objective was to assess the presence of a reduced inhibitor activity or an increased promoter activity in urine of IUASF compared to non UASF, independent of urinary pH.

METHODS

Study Participants

The study was approved by the Institutional Review Board at the University of Texas Southwestern Medical Center (UTSW, Dallas, TX), and all participants provided informed

consent. Three groups were enrolled for this study: idiopathic uric acid stone formers (IUASF), obese non-stone formers (NSF) ($\text{BMI} > 30 \text{ kg/m}^2$) and healthy lean NSF ($\text{BMI} < 25 \text{ kg/m}^2$). IUASF were defined by 100% UA stones (by stone composition) with no identifiable primary disorder such as gout, chronic diarrhea, neoplasms, or known genetic mutations.¹⁸ NSF were defined by the absence of a personal history of stone disease. Patients were recruited from the UTSW-affiliated hospitals and clinics, the Dallas Veterans Affairs Medical Center, and through local advertisements. Subjects were of either sex, any ethnicity, and age ≥ 21 years. We excluded pregnant women and individuals with T2D, chronic diarrheal illness, previous bariatric surgery, creatinine clearance $< 60 \text{ mL/min/1.73 m}^2$, or liver disease. Patients on allopurinol, alkali therapy or diuretics discontinued these medications for one week before evaluation.

Study Protocol

The study duration was 5 days [4 outpatient days and 1 inpatient day in the UTSW Clinical and Translational Research Center (CTRC)]. Subjects were maintained on a frozen metabolic diet (55% calories from carbohydrates, 30% from fat, and 15% from protein) with a constant daily intake of 400 mg calcium, 800 mg phosphorus, 100 mEq sodium, and 3000 cc distilled water. Subjects were then admitted to the inpatient CTRC on the morning of day 5 and provided a 24-hour urine collection while consuming the same metabolic diet. Urine samples were kept refrigerated with no preservative during collection, and processed within 24 hours for measurement of total volume, pH, sodium, potassium, calcium, magnesium, phosphorus, chloride, oxalate, citrate, uric acid, ammonium and creatinine.

Crystallization experiments in urine to assess for reduced inhibitor activity or increased promoter activity independent of pH were conducted at a constant pH of 5.0. Based on methods previously used for the study of CaOx crystallization^{19–24}, three parameters were assessed: spontaneous nucleation (SN, representing *de novo* crystal formation), crystal growth (CG, representing growth of UA crystals when a small quantity of UA crystals is added to urine) and steady state of UA solubility (SS UA, measured as the maximum amount of UA able to dissolve in urine when an excess of UA crystals is added to urine).

For these experiments, a fresh aliquot of whole urine was obtained at the end of the 24-hour urine collection, centrifuged to remove crystals and cellular debris, with the supernatant filtered through a $0.22 \mu\text{m}$ filter to eliminate any remaining crystalline material. Urine was then titrated to pH 5.0 with a dilute hydrochloric acid solution, and incubated at 37°C for 6 hours under constant stirring with a magnetic bar, with urine pH checked hourly and maintained at 5.0.

To assess for spontaneous UA nucleation, urine aliquots were simply maintained at pH 5.0 for 6 hours under constant stirring. At 0, 3 and 6 hours, 2 mL aliquots of suspension were removed and filtered through $0.22 \mu\text{m}$ Millipore filter and UA concentration ([UA]) was measured in the filtrate. A decline in [UA] in filtrates from time 0 and subsequent time points represents UA SN. For crystal growth (CG), a seed of solid anhydrous UA at the concentration of 0.1 mg/mL was added to a separate urine aliquot. When seeded by 0.1 mg/mL UA, [UA] declines slowly, reaching a nadir in 6 hours. The filtrates before and after incubation with UA seed were analyzed for [UA], with the change in [UA] from hour 0

indicating CG. For steady state solubility of UA (SS UA), an excess of UA (5 mg/mL) was added to a separate urine aliquot. After adding an excess of UA, [UA] declines rapidly, reaching a nadir in 6 hours. This 6-hour concentration represents SS UA.²⁴ For these three physicochemical parameters, an inhibitor activity should result in lower changes in [UA], while promoter activity should result in a greater decrease in [UA]. Supersaturation index of Uric Acid (SI UA) was determined by the Joint Expert Speciation System (JESS, Mayhem Unit Trust and Council for Scientific and Industrial Research, Pretoria, South Africa) software, version 6.5 on baseline 24-hour urine and at time 0.²⁵

Analytical Procedures

The methodology for urine biochemistry has been previously described in detail.³ Briefly, urinary pH was measured with a pH electrode, and UA by the urate oxidase method.

Statistical Analyses

Results are presented as median with interquartile range or as mean $\pm$ SD. The Kruskal-Wallis test was used to compare continuous variables between the study groups, the Mann-Whitney test for pairwise comparisons, and the Wilcoxon rank sum test for repeated measures in a single group. All tests were conducted using the *R* Software, version 3.2.2. A *p*-value ≤ 0.05 was considered significant.

RESULTS

Demographics

A total of 51 patients were enrolled in the study: 30 IUASF, 9 obese NSF and 12 lean NSF. There was no significant difference between groups in the gender distribution (*p*=0.72), age (*p*=0.63) and height (*p*=0.67) (Table 1). Body weight and BMI were significantly higher in the IUASF and obese NSF groups than the lean NSF group, *p*<0.05 (Table 1).

Baseline 24-h urine parameters

After equilibration on the fixed metabolic diet, 24-hour urinary pH was significantly lower and baseline SI UA significantly higher in IUASF than NSF, *p*<0.05 (Table 2 and Figure 1). There was no significant difference between the three groups in UA content. IUASF had significantly higher urinary phosphorus and creatinine compared with lean NSF (Table 2).

Crystallization Studies

At time 0 (*T*=0), when pH was dropped to 5.0, no significant change in [UA] was observed compared to the baseline values. However, at pH of 5.0, SI UA became significantly higher in all groups compared to the baseline values, *p*<0.05. Median SI UA at *T*=0 was 2.8 in IUASF, 3.2 in obese NSF and 2.2 in lean NSF with a significant difference between obese and lean NSF, *p*=0.005 (Table 3).

Spontaneous nucleation—UA concentration did not change significantly over 6 hours in the three groups, suggesting lack of SN at pH=5.0 (Figure 2A). The respective changes between *T*=0 and 6 hours were −2.5, −1, and −0.5 mg/L in the IUASF, obese NSF and lean NSF, *p*=0.76 (Table 3).

Crystal growth—Compared to T=0, UA concentration dropped significantly in the three groups at 3 and 6 hours ($p<0.05$) after the addition of a 0.1 mg/mL UA seed, suggesting CG occurred in all groups (Figure 2B). The drop was significantly higher in the obese NSF group than IUASF and lean NSF at 6 hours ($p=0.049$ and $p=0.03$, respectively) (Table 3).

Steady state solubility of UA—Upon addition of 5 mg/mL UA seed, UA concentration dropped significantly in the three groups, $p<0.05$ (Figure 2C). Median steady state concentration at 6 hours was similar in all 3 groups: 111, 113 and 106 mg/L in IUASF, obese NSF and lean NSF respectively, $p=0.18$ (Table 3).

Relationship between crystal growth and baseline SI UA—Figure 3 shows the relationship between SI UA at time 0 and CG depicted as change in [UA] between time 0 (closed symbol) and 6 hours (open symbol) in individual subjects according to study group. Across all study groups, a higher SI UA at time 0 is associated with a greater fall in [UA] indicating greater CG. Since pH was kept constant at 5.0, the higher SI UA represents a higher UA concentration at time 0.

DISCUSSION

This is the first study comparing urinary crystallization parameters in IUASF vs. obese and lean NSF with the primary aim of uncovering the presence of promoter or inhibitor activity that may affect UA stone formation independent of urinary pH. The three groups were examined using urine specimen collected under carefully controlled dietary conditions, and experiments were conducted at 37°C, and at a fixed pH of 5.0. We found that no significant SN occurred in the three groups. There were small but significant differences in UA CG between the groups which were likely driven by baseline differences in [UA] and SI UA. Finally, SS UA was similar between IUASF and NSF. Overall, our findings suggest that there is no significant difference between IUASF and NSF in terms of promoter or inhibitor activity against UA stone formation at low urinary pH and that UA stone formation is primarily driven by the degree of urinary UA saturation.

Findings and interpretations—In this study, 24-hour urinary pH was lower in IUASF compared to the NSF groups, consistent with the published literature.^{3,18} The lack of significant difference in 24-hour urinary pH between obese and lean NSF under metabolic diet (pH=5.93 and pH=6.08 respectively, $p=0.6$) may be related to the small sample size in these two groups.

To assess the potential effect of inhibitor or promoter activity, we evaluated three different parameters essential for lithogenesis: spontaneous nucleation, crystal growth and steady state of UA solubility. SN is *de novo* crystal formation and leads to a decline in UA concentration in urine from which formed crystals are filtered. When SN occurs, the new crystals created are a seed for further crystal growth, which leads to the adsorption of urinary UA ions to the crystals. To assess for CG, a “seed” crystal of anhydrous UA was added to start the growth. Finally, the SS UA solubility assesses the maximum amount of UA that can dissolve in urine, and was tested experimentally by adding an excess of UA to a urinary aliquot.

We found that no substantial SN occurred in the three groups, as there was no significant change in [UA] over six hours (Table 3). Although some SN occurred in a handful of patients (Figure 2A), the frequency and magnitude was not different between IUASF and NSF. In regards to CG, a similar drop in [UA] was observed in all groups after 3 hours. At 6 hours, the decrease in [UA] was significantly higher in the obese NSF than the lean NSF group and at the limit of significance compared to the IUASF ($p=0.049$), while no difference was found between IUASF and lean NSF. Although results in obese NSF may suggest increased promoter or reduced inhibitor activity, these findings need to be interpreted with caution since [UA] and urinary saturation (assessed as SI UA) in this group were significantly higher than in the lean NSF and nominally higher than the IUASF at the start of the different experiments (at $\text{pH}=5.0$, at *time 0*). The SS UA was similar in all 3 groups of patients, making it unlikely that inhibitors or promoters can alter maximum UA solubility at a pH of 5.0. Furthermore, the steady-state values approximated the solubility of UA in urine (100 mg/L) at pH 5.0. Another finding of our experiments is the linear dependence of CG on baseline SI UA under experimental conditions (Figure 3), suggesting that in a physiologic state, crystal growth is more likely to occur in IUASF who have higher 24-hour urine SI UA.

Strengths and limitations—This is the first study reporting the feasibility of reliable methods assessing UA crystallization in whole urine. 51 subjects were equilibrated for 5 days on a fixed metabolic diet. The number of participants included in each group was relatively small, but sufficient to provide a power of 80% to detect a mean net difference of 30 mg/L in [UA] in the three urinary crystallization parameters evaluated. The constant metabolic diet was designed to control for variability in dietary intake across individuals and limit the impact of variation in major urinary anions and cations on UA crystallization. However, the crystallization studies were conducted on urinary samples that may not be representative of the subjects' typical urinary environment on a free-choice diet. Another limitation is that all crystallization experiments were conducted at a constant pH of 5.0 to assess for pH-independent risk factors for UA stone formation. However, this low pH may have altered the structure and/or function of putative urinary promoters and inhibitors of UA crystallization. Another explanation of our results could be that the inhibitory or promoter activity against UA crystallization occurs earlier in the urinary tract and may not be captured in whole urine. An example of promoter activity could be that nucleated crystals aggregate to particles too large to pass freely through the tubules or because crystals become abnormally adherent to tubular cell surface.^{26,27} On the other hand, inhibitor or promoter activity of UA stone formation may impact later steps in the process of stone formation such as crystal aggregation²⁸, which our studies were not set up to assess. Furthermore, some promoters or inhibitors may have been removed after centrifugation of fresh aliquot of whole urine to remove crystals and cellular debris. Finally, although IUASF and lean NSF groups had similar [UA] and SI UA at the start of the experiments, these parameters were higher in the obese NSF, which may have contributed to the slight differences in the results of the CG experiments the obese NSF group compared to the two other groups. Further studies are therefore needed to assess the potential role of promoters or inhibitors of UA stone formation, but would need to be conducted at different pH levels and potentially with

similar starting SI UA. The physicochemical methods used in this study could serve as basis for such future experiments.

CONCLUSIONS

Overall, our findings suggest that there is no significant difference between IUASF and NSF in terms of promoter or inhibitor activity against UA stone formation at a constant pH in whole urine. The findings suggest that the formation of UA stones is dictated by a high urinary saturation with respect to UA, driven primarily by a low pH.

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Key of Definitions for Abbreviations

UA	Uric acid
CaOx	Calcium Oxalate
IUASF	Idiopathic uric acid stone formers
NSF	Non-stone formers
SN	Spontaneous Nucleation
CG	Crystal growth
SS UA	Steady-state Solubility of Uric Acid
SI UA	Supersaturation Index with respect to Uric Acid

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Figure 1.
Distribution of Supersaturation Index for uric acid (SI UA) at baseline (24-hr urine) in each of the three groups.

Figure 2.

Evolution of uric acid concentration during spontaneous nucleation, crystal growth and steady state of uric acid solubility at time 0, 3 and 6 hours.

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Figure 3.

Relationship between baseline Supersaturation Index for uric acid (SI UA) and change in uric acid concentration during the crystal growth experiments. Closed symbols represent uric acid concentration at time 0 and open symbols represent uric acid concentration at 6 hours.

Table 1

Demographic data.

	IUASF	Obese NSF	Lean NSF	<i>p</i> value (by Kruskal-Wallis Test)
Number of subjects	30	9	12	
Height, cm	171 ± 9	168 ± 7	171 ± 12	0.67
Body weight, kg	109.7 ± 30.2	112.3 ± 23.9	66.2 ± 6.8 [*] , [#]	< 0.001
Body mass index, kg/m ²	35.4 [31.7 ; 46.4]	40.3 [32.6 ; 46.6]	23.6 [20.5 ; 24.7] [*] , [#]	< 0.001
Age, yr	56.3 ± 11.6	57.6 ± 9.7	54.3 ± 9.5	0.63
Sex, men/women	19 / 11	5 / 4	6 / 6	0.72
Race, white/asian/black	24 / 5 / 1	6 / 3 / 0	8 / 4 / 0	0.62

IUASF = Idiopathic Uric Acid Stone Formers; NSF = Non-Stone Formers

Data shown as Mean ± standard deviation or Median [25th percentile; 75th percentile]^{*} Statistical significance by pairwise comparison for IUASF vs. obese NSF or lean NSF groups (*p* < 0.05)[#] Statistical significance by pairwise comparison for obese NSF vs. lean NSF groups (*p* < 0.05)

Table 2

Baseline 24-hour urine biochemistry.

	IUASF	Obese NSF	Lean NSF	<i>p</i> value (by Kruskal-Wallis Test)
Total volume, liters	2.5 ± 0.6	2.5 ± 0.7	2.6 ± 0.5	0.76
pH	5.43 [5.14 ; 5.78]	5.93 [5.72 ; 6.54] *	6.08 [5.86 ; 6.55] *	< 0.001
Uric acid, mg/day	557 ± 186	636 ± 226	486 ± 122	0.2
Citrate, mg/day	546 [284 ; 789]	497 [336 ; 568]	517 [397 ; 736]	0.84
Ammonium, mEq/day	29 ± 10	33 ± 12	28 ± 5	0.54
Phosphorus, mg/day	664 ± 194	577 ± 178	523 ± 159 *	0.06
Sodium, mEq/day	91 ± 34	92 ± 30	92 ± 37	0.92
Potassium, mEq/day	43 ± 10	42 ± 14	40 ± 9	0.87
Calcium, mg/day	101 ± 51	104 ± 56	133 ± 82	0.43
Oxalate, mg/day	29 ± 6	29 ± 8	25 ± 4	0.22
Chloride, mEq/day	94 [71 ; 107]	106 [71 ; 110]	87 [74 ; 111]	0.9
Magnesium, mg/day	91 ± 31	102 ± 47	97 ± 27	0.81
Creatinine, mg/day	1531 ± 380	1437 ± 651	1141 ± 312 *	0.02
SI UA	1.75 ± 1.94	0.84 ± 0.53 *	0.57 ± 0.35 *	< 0.001

IUASF = Idiopathic Uric Acid Stone Formers; NSF = Non-Stone Formers

Data shown as Mean ± standard deviation or Median [25th percentile; 75th percentile]* Statistical significance by pairwise comparison for IUASF vs. obese NSF or lean NSF groups (*p* < 0.05)# Statistical significance by pairwise comparison for obese NSF vs. lean NSF groups (*p* < 0.05)

Table 3

Results of the crystallization experiments.

	IUASF	Obese NSF	Lean NSF	<i>p</i> value (by Kruskal-Wallis Test)
<i>Time 0-h (pH dropped to 5.0)</i>				
pH (T=0)	5.00 [5.0 ; 5.0]	5.00 [5.0 ; 5.0]	5.00 [4.98 ; 5.0]	0.66
[UA] (T=0)	213 [173 ; 269]	242 [199 ; 313]	176 [163 ; 213] [#]	0.03
SI UA (T=0)	2.8 [2.2 ; 3.5]	3.2 [2.6 ; 4.0]	2.2 [2.1 ; 2.8] [#]	0.02
<i>Spontaneous Nucleation</i>				
[UA] (T=3h)	-0.5 [-4 ; 4]	-2.0 [-7.5 ; 1]	-0.5 [-3.5 ; 3.75]	0.74
[UA] (T=6h)	-2.5 [-7 ; 1.5]	-1 [-12 ; 2.5]	-0.5 [-5 ; 2.5]	0.76
<i>Crystal Growth</i>				
[UA] (T=3h)	-23.5 [-57 ; -6.0]	-40 [-66 ; -19.5]	-17 [-29.5 ; -1.5]	0.25
[UA] (T=6h)	-28 [-70 ; -10]	-66 [-91 ; -32.5] [#]	-28 [-47.5 ; -6] [#]	0.06
<i>Steady State of UA solubility</i>				
[UA] (T=3h)	117 [105 ; 144]	118 [108 ; 127]	108 [99 ; 120]	0.34
[UA] (T=6h)	111 [103 ; 123]	113 [103 ; 128]	106 [88 ; 112]	0.18

IUASF = Idiopathic Uric Acid Stone Formers; NSF = Non-Stone Formers

Spontaneous nucleation = de novo crystal formation

Crystal growth = change in [UA] in response to addition to low concentration of added UA crystals (seed of 0.1 mg/mL UA) Steady state of UA solubility = change in [UA] in response to addition to high concentration of added UA crystals (seed of 5 mg/mL UA)

[UA] = UA concentration in mg/L

[UA] = Difference in UA concentration between times T=3h/T=6h and initial concentration at T=0

SI UA = Supersaturation Index with respect to UA calculated by JESS 6.5

Data shown as Median [25th percentile; 75th percentile]* Statistical significance by pairwise comparison for IUASF vs. obese NSF or lean NSF groups ($p < 0.05$)[#] Statistical significance by pairwise comparison for obese NSF vs. lean NSF groups ($p < 0.05$)