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Urinary Tamm-Horsfall Protein and Citrate: A Case-Control Study of Inhibitors and Promoters of Calcium Stone Formation

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ABSTRACT

Introduction: This study aimed to compare urinary Tamm-Horsfall protein (THP), citrate, and other inhibitors and promoters of stone formation in calcium stone formers with those in healthy individuals.

Materials and Methods: From January 2002 to June 2004, 100 calcium stone formers (mean age, 38.6 ± 10.3 years) who had at least 2 episodes of calcium stone formation were compared with 100 healthy individuals (mean age, 33.8 ± 9.7 years). Their 24-hour urine THP (using the sodium dodecyl sulfate polyacrylamide gel electrophoresis method), citrate, calcium, uric acid, oxalate, and magnesium values were measured and compared.

Results: The mean 24-hour urine THP was 3.3 ± 8.1 mg in patients in the study group and 4.6 ± 19.2 mg in controls ($P = 0.5$). However, THP in individuals with and without bacteriuria was significantly different (15.8 ± 33.6 versus 2.6 ± 10.2, $P < 0.001$). Mean 24-hour urinary calcium, citrate, and oxalate values were 232.6 ± 95.3 mg and 177.8 ± 82.7 mg ($P < 0.001$), 132 ± 103.2 mg and 395 ± 258.5 mg ($P < 0.001$), and 18.9 ± 22.5 mg and 10.4 ± 8.5 mg ($P < 0.001$) in patients in the study and control groups, respectively. There was a significant positive correlation between urinary citrate and promoters of stone formation, including urinary calcium, oxalate, and uric acid, in patients in the control group, but not in patients in the study group.

Conclusion: THP in the urine of stone formers is not quantitatively different from that of healthy individuals, but it is different in patients with bacteriuria. Increased urinary excretion of calcium, oxalate, and uric acid in stone formers with no increase in urine citrate may play a role in the pathogenesis of recurrent stone formation.

KEY WORDS: calcium stone formation, Tamm-Horsfall protein, citrate, metabolic abnormalities

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Introduction

Urinary stones have been recognized since ancient times. The prevalence of urinary stone disease is estimated to be between 4% and 9% in males and between 1.7% and 4.1% in females, and the likelihood of developing stone disease in a white man by age 70 is about 1 in 8.[1] The recurrence rate for patients with calcium oxalate renal stones—without treatment—is about 10% to15% at 1 year, 35% to 50% at 5 years, and 50% to 60% at 10 years.[2,3] Unfortunately, laboratory evaluation cannot discern patients who will have stone recurrence from those who will not.[4] One study has shown that about 20% of patients with recurrent stone disease who underwent surgery for obstruction and infection, developed mild renal insufficiency.[5] The etiology of stone formation is one of the important issues in urologic research. Measuring urinary inhibitors and promoters of stone formation can be helpful, not only in establishing medical treatment protocols, but also in predicting the probability of stone formation in individuals with a positive family history.

The Tamm-Horsfall protein (THP), synthesized in the renal thick ascending limb of Henle's loop and distal tubule, is the most potent aggregation inhibitor identified to date.[6] In addition, THP may incorporate into the stone matrix.[7] Thus measurement of urinary THP levels and their correlation with metabolic abnormalities may help shed light on THP's role in stone formation. Citrate is the most important ion that binds to calcium in urine and reduces ionic calcium concentration.[8] Hypocitraturia is considered a major correctable cause of calcium oxalate nephrolithiasis and has been reported in 15% to 63% of patients with nephrolithiasis.[8] This study sought to determine the role of urinary proteins and other risk factors to better understand the pathogenesis of calcium stone formation.

Materials and Methods

From January 2002 to June 2004, 100 consecutive patients at Sina Hospital in Tehran, Iran, were enrolled in this cross-sectional case-control study. There were 70 men and 30 women, aged 38.6 ± 10.3 years (range, 20 to 50 years), who had at least 2 episodes of calcium (calcium oxalate and/or calcium phosphate) stone formation documented by imaging evaluations (intravenous urography, KUB, ultrasonography) and stone analysis. Patients with a history of endocrine diseases (hyper and hypoparathyroidism, hyper and hypothyroidism, and diabetes mellitus) were excluded. Also, patients with a history of urologic intervention or renal colic over the 4 weeks prior to the evaluations, as well as those with test results showing impaired kidney function were excluded. Medications known to interfere with metabolism of calcium, oxalate, citrate, uric acid, magnesium, or phosphate were discontinued at least 2 weeks prior to the evaluation. None of the female patients were menopausal or pregnant.

For comparison, 100 sex- and age-matched healthy individuals (67 men and 33 women, aged 33.9 ± 9.7 years) without renal stones (as confirmed by urinalysis and ultrasonography) volunteered to participate in our study.

All the patients and controls underwent ambulatory metabolic evaluations, while adhering to their free-choice diet. Serum sampling for creatinine and BUN levels was performed, as was a random urine test for bacterial culture, and a 24-hour urine collection. The 24-hour urine collection was refrigerated. Urine volume was measured and divided into samples for uric acid, pH, and urine protein electrophoresis. HCl was added as conservative. Then urinary sodium, potassium, calcium, oxalate, citrate, phosphate, and magnesium values were measured. Patients and controls who had collected 24-hour urine specimens in an incorrect manner (according to 24-hour creatinine) were excluded from the study.

Methods used for measuring each of the metabolites were as follows: methylthymol blue method for calcium, phenol-aminophenazone peroxidase method for uric acid, xylidyl blue for magnesium, UV method with molybdate for phosphorus, standard method for sodium and potassium, and enzymatic method for oxalate and citrate measurement.

Twenty-four-hour urinary levels of proteins were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE method). Concentration of urine protein was assessed measured by the Bradford method.[9] SDS-PAGE of unconcentrated urine protein was performed on a vertical discontinuous gradient gel (stacking part 4%, resolving part 6%, 11%, and 15%).[10] Polyacrylamide gels (0.6 mm thick) were cast between 12 × 12 cm glass plates. Each urine sample (20 μL) was incubated at 100°C for 3 minutes with 20 μL of sample buffer (4% SDS,

Table 1. *Main fractionated urinary proteins (mg/24 h) in controls and study patients as measured by urine protein electrophoresis (SDS-PAGE method), n = 100 in each group (rows in ascending order, according to molecular weight).*

Parameters	Controls	Subjects	*P* value
24-hour urine beta 2-microglobulin	0.0 ± 0.0	0.27 ± 2.67	0.31
24-hour urine hemoglobin	0.0 ± 0.0	0.29 ± 2.85	0.31
24-hour urine light chain	0.0 ± 0.0	0.18 ± 1.27	0.16
24-hour urine apoprotein A1	0.0 ± 0.0	0.55 ± 2.96	0.066
24-hour urine alpha 1-microglobulin	0.0 ± 0.0	0.75 ± 4.42	0.08
24-hour urine albumin	74.26 ± 46.05	163.31 ± 151.92	< 0.001
24-hour urine transferrin	0.40 ± 3.56	8.09 ± 27.59	0.006
24-hour urine THP	4.63 ± 19.24	3.35 ± 8.15	0.53
24-hour urine immunoglobulin G	0.0 ± 0.0	19.45 ± 110.97	0.08
24-hour urine immunoglobulin M	0.0 ± 0.0	1.16 ± 7.44	0.12

All values are shown as mean ± SD

0.125 M Tris HCl, pH 6.8, 20% glycerol, 0.01% bromophenol blue) before electrophoresis. Then 40 μL of each sample was applied on the gel using a Hamilton syringe. SDS-PAGE was run at 30 mA for 2.5 hours. Silver staining was performed according to fast silver staining protocol.[11]

All values are presented as means ± SD. The SAS system version 8.00 was used for statistical analyses. Pearson product moment correlation coefficient was used to test the relationship between the variables, and analysis of variance and Friedman tests were used for group comparisons. A *P* value less than 0.05 was considsred statistically significant.

Results

The concentrations of fractionated proteins in the urine samples of patients in the study and control groups are shown in Table 1. Of urinary proteins, only albumin and transferrin levels were statistically higher in patients in the study group; the differences of THP, alpha-1 microglobulin, and beta-2 microglobulin levels were not statistically significant. However, there was a significant difference between urinary excretion of THP among individuals with and without bacteriuria, both in patients in the study group and in controls (Table 2).

Urinary parameters in the study and control groups are shown in Table 3. In 24-hour urine, only calcium, oxalate, and citrate values were statistically different between the two groups. Comparing the mean values of metabolites and urinary proteins, there were no significant differences between calcium oxalate and calcium phosphate stone formers; however, there was a significant difference between their 24-hour urine pH values (5.2 in calcium oxalate stone formers versus 7.3 in calcium phosphate cases, $P < 0.001$).

The correlations between each urinary metabolite and other parameters in controls and study subjects are shown in Tables 4 and 5; there were significant positive correlations between urine citrate, and urine calcium, uric acid, oxalate, and magnesium in our controls, but not in our study subjects.

Discussion

To our knowledge, this is the largest case-control study of urinary protein excretion and urinary metabolites in Iranian renal calcium stone formers. However, concerning calcium oxalate stone formation, there is no clear-cut factor to differentiate the population of stone-formers from healthy individuals. Calcium stone

Table 2. *Mean THP in individuals with and without bacteriuria (mg/24 h).*

Group	Individuals Without bacteriuria	Individuals With bacteriuria	*P* value
Control group	3.00 ± 13.54 (n = 93)	26.22 ± 52.04 (n = 7)	0.002
Study group	2.36 ± 4.74 (n = 87)	10.19 ± 18.13 (n = 13)	0.009
Total	2.67 ± 10.25 (n = 180)	15.80 ± 33.53 (n = 20)	0.001

All values are shown as mean ± SD. Bacteriuria: more than 100,000 colony-forming units per milliliter

TABLE 3. *Urinary metabolites (mg/24 h) in controls and study subjects, n = 100 in each group.*

Metabolic parameters	Controls	Subjects	*P* value
24-hour urine sodium	190.3 ± 90.19	182.46 ± 74.7	0.05
24-hour urine potassium	47.56 ± 39.07	42.44 ± 32.98	0.31
24-hour urine calcium	177.80 ± 82.87	232.59 ± 95.3	< 0.001
24-hour urine phosphorus	572.19 ± 251.13	639.79 ± 234.30	0.05
24-hour urine uric acid	470.88 ± 178.78	490.95 ± 234.42	0.49
24-hour urine citrate	395.01 ± 258.47	131.95 ± 103.20	< 0.001
24-hour urine oxalate	10.41 ± 8.5	18.90 ± 22.48	< 0.001
24-hour urine magnesium	101.13 ± 46.19	100.25 ± 39.21	0.88
24-hour urine volume	1252.31 ± 559.41	1495.85 ± 671.93	< 0.001
24-hour urine pH	5.85 ± 0.82	5.31 ± 0.67	< 0.001

All values are shown as mean ± SD.

TABLE 4. *Correlation between inhibitors and promoters of calcium stone formation in control group (in each cell, upper row shows Pearson correlation coefficient and lower row shows P value).*

	Urine calcium	Urine oxalate	Urine uric acid	Urine magnesium	Urine citrate	Urine THF
Urine calcium		0.13 (0.19)	0.39 (< 0.001)	0.56 (< 0.001)	0.23 (0.02)	-0.06 (0.545)
Urine oxalate	0.13 (0.19)		0.38 (< 0.001)	0.13 (0.20)	0.23 (0.02)	0.02 (0.86)
Urine uric acid	0.39 (< 0.001)	0.38 (< 0.001)		0.46 (< 0.001)	0.21 (0.04)	0.05 (0.65)
Urine magnesium	0.56 (< 0.001)	0.13 (0.20)	0.46 (< 0.001)		0.33 (< 0.001)	0.125 (0.21)
Urine citrate	0.23 (0.020)	0.23 (0.02)	0.21 (0.04)	0.33 (< 0.001)		-0.09 (0.37)
Urine THP	-0.06 (0.545)	0.02 (0.86)	0.05 (0.65)	0.125 (0.21)	-0.09 (0.37)	

TABLE 5. *Correlation between inhibitors and promoters of calcium stone formation in case group (in each cell, upper row shows Pearson correlation coefficient and lower row shows P value).*

	Urine calcium	Urine oxalate	Urine uric acid	Urine magnesium	Urine citrate	Urine THF
Urine calcium	1.00	0.03 (0.790)	0.39 (< 0.001)	0.335 (< 0.001)	0.05 (0.63)	0.09 (0.37)
Urine oxalate	0.03 (0.79)	1.00	-0.03 (0.73)	0.20 (0.05)	0.04 (0.68)	-0.02 (0.85)
Urine uric acid	0.39 (< 0.001)	-0.03 (0.73)	1.00	0.39 (< 0.001)	0.08 (0.44)	0.22 (0.02)
Urine magnesium	0.335 (0.00)	0.20 (0.05)	0.39 (< 0.001)	1.00	-0.10 (0.31)	0.01 (0.93)
Urine citrate	0.04 (0.68)	0.04 (0.68)	0.08 (0.44)	-0.10 (0.31)	1.00	0.4 (0.68)
Urine THP	-0.02 (0.85)	-0.02 (0.85)	0.22 (0.02)	0.01 (0.93)	0.4 (0.68)	1.00

formation is unlikely to have a single cause, and a combination of risk factors should be considered.[12]

In the present study, mean THP levels in study patients and in controls were not statistically different. In general, available studies suggest no difference in urinary THP excretion between kidney stone formers and nonstone formers.[13-16] Nevertheless, in some studies, urinary THP excretion was reduced in stone formers or their subgroups.[17-20]

Glauser and coworkers showed decreased excretion of THP in calcium stone formers and its correlation with stone forming ions, calcium and oxalate, in healthy individuals.[17] Ganter and coworkers found reduced THP and citrate excretion in calcium oxalate stone-forming patients and indicated a tubular dysfunction of the distal section.[18] Bichler and colleagues showed lower THP excretion in patients with uric acid urinary stones. Nevertheless, they expressed that the role of THP is still unclear. It is unknown whether it acts as a protector, an inhibitor, a promoter, or even as a direct transporter.[19] It has been suggested that in highly concentrated urine, THP polymerizes readily to such an extent

that it overwhelms the inhibitors in urine and strongly promotes agglomeration of calcium oxalate monohydrate crystals.[21] Our study showed that THP level as an inhibitor of stone formation does not differ quantitatively. Boeve and coworkers showed that THP particles are smaller and have different electrical potential in normal subjects compared with stone formers. They concluded that differences in molecular structures may cause functional differences in the ability of THP to inhibit aggregation. They emphasized that research on the role of THP in stone formation should not be restricted to the urinary environment only, and that understanding the role of THP at a cellular level in the early stage of stone formation could be very useful.[22]

Results of Tardivel and colleagues' study showed that urinary alpha-1 microglobulin was significantly lower in calcium oxalate stone formers. They concluded that this protein could influence the risk of crystallization in vivo.[23] Pupek-Musialik found higher beta-2 microglobulin excretion in the urine of patients with metabolically active urolithiasisstone formers. They suggested a dysfunction of the proximal tubule in stone formers.[24] Our study did not show a statistical difference between study subjects and controls with regard to urinary alpha-1 microglobulin and beta-2 microglobulin.

There was a statistically significant difference in the albumin and transferrin means between study subjects and controls. Of note, the higher level of albumin and transferrin found in the 24-hour urine specimens of stone formers is a new finding. Indeed, we do not expect glomerular impairment in stone formers, but we speculate that urinary albumin and transferrin may serve as a nidus for crystallization. Using the SDS-PAGE method, Siddiqui and colleagues isolated several proteins (THP, albumin, and transferrin) from urinary stones. Because the same proteins are present in the urine of stone formers in high concentrations, but not or only to a very minor extent in the urine of nonstone formers, the authors concluded that they are selectively incorporated into renal stones, and that they most likely have a role in creating a nidus and therefore, in early stone formation.[25] Similar results have been found by Faraij.[26] However, Chen and coworkers have shown the in vitro inhibitory effect of albumin on crystallization.[27] The exact roles of albumin and transferrin remain to be elucidated.

Significant differences between THP means in individuals with and without bacteriuria have been previously reported in the literature.[28,29] The normal physiologic function of THP remains elusive, however, despite extensive studies. The biochemical properties of THP make it possible that a host defense factor might be involved in clearing bacteria from the urinary tract. Bates and colleagues have shown that THP serves as a soluble receptor for type 1 fimbriated E. coli and helps eliminate bacteria from the urinary tract .[29] Mo and coworkers provided evidence clearly establishing THP on the first line of host defenses against both renal stone formation and bacterial infection.[30]

How the renal epithelium responds to hyperoxaluria or calcium oxalate crystals might prove to be a contributing factor in the development of clinical urolithiasis.[31] The significant positive correlation between urine citrate and urinary promoters of stone formation (urinary uric acid, oxalate, and calcium) in controls but not in study patients suggests the probability that increased urinary excretion of citrate in response to elevated levels of stone formation promoters is a protective response. This might explain why the high frequency of metabolic abnormalities in patients in the control group did not lead to stone formation. We hypothesize that impairment of this response in stone formers might predispose them to stone formation. Boruczkowska found this correlation in patients with urolithiasis.[32]

In this study, in 24-hour urine specimens, only the mean levels of calcium, oxalate, and citrate were statistically different between the two groups. For the past 25 years, it has been recognized that other than a reduction in urine volume, increased urinary excretion of oxalate is a major risk factor for calcium oxalate stone formation.[12] Hypercalciuria appears to play, at most, only a secondary role in the genesis of calcium stones compared with mild hyperoxaluria.[33]

We can now hypothesize that decreased excretion of citrate and impairment of its increase in response to elevated levels of urinary promoters of stone formation might be another important risk factor.

The higher 24-hour urine volume found in stone formers (which was statistically significant although not large enough to prevent stone

formation) might be due to greater liquid consumption by patients who considered the advice of health practitioners.

Conclusion

THP in the urine of calcium stone formers is not quantitatively different from that of healthy individuals, but it increases in with the presence of bacteriuria. Albumin and transferrin were significantly higher in the urine of calcium stone formers, suggesting their role in matrix and stone formation. Increased urinary excretion of calcium, oxalate, and uric acid in stone formers with no increase in urinary citrate might play a role in the pathogenesis of recurrent stone formation. Being able to control this predisposing factor would undoubtedly constitute a major breakthrough in preventing recurrence of calcium oxalate stones.

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Editorial Comment

The authors have reapproached the long-standing debate on whether or not THP is implicated in calcium stone formation. Over 130 peer-reviewed papers have been published so for on this subject with no unified consensus. Part of this ambiguity is due to the fastidious nature of THP assays. We found, for example, that urine samples must be processed within 4 hours of collection or instantly frozen over liquid nitrogen, otherwise, any slower freeze-thaw cycle would lead to stubborn uromucoid aggregation and confound the results of future analyses.[1] Despite these efforts to curb interfering factors, their samples were collected on an ambulatory basis from patients on an uncontrolled diet. Dietary Ca, P, and Mg load each have been proven to grossly influence urinary THP excretion.[2] This may have played a significant role in the present results. Even given a constant level of THP, Sumitra and coworkers recently showed that antiaggregation and antinucleation effects of the uromucoid are radically influenced by the antioxidant content of the daily diet.[3] Therefore, while commending the authors on such hard-won results, one must consider keeping such confounding elements in mind.

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Reply by Author

Ambulatory evaluation is routine in THP studies as mentioned in the literature (references 17-19, 25, and 26 of the article). Besides, we should consider that effect of confounding factors can be overwhelmed by designing a case-control study, in which two groups are matched.

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