

Lime powder treatment reduces urinary excretion of total protein and transferrin but increases uromodulin excretion in patients with urolithiasis

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Received: 6 March 2017 / Accepted: 25 May 2017 / Published online: 5 June 2017
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Abstract Our previous study has shown that lime powder (LP) had an inhibitory effect against calcium oxalate stone formation. However, the precise mechanisms underlying such beneficial effect remained unclear. Our present study thus aimed to address the effect of LP on excretory level and compositions of urinary proteins using a proteomics approach. From a total of 80 calcium oxalate stone formers recruited into our 2-year randomized clinical trial of LP effect, 10 patients with comparable age and clinical parameters were selected for this proteomic study. 24-h urine specimens were collected from all subjects, at baseline (before) and after LP treatment for 6 months, and then subjected to quantitative proteomics analysis and subsequent validation by ELISA. Total urinary protein excretion was significantly decreased by LP treatment, but unaffected

by placebo. Nanoflow liquid chromatography coupled to tandem mass spectrometry (nanoLC–MS/MS) followed by quantitative analysis revealed 17 proteins whose levels were significantly altered (16 decreased and 1 increased) exclusively by LP treatment. Among these, the decrease of transferrin and increase of uromodulin were validated by ELISA. Moreover, there was a significant correlation between microalbuminuria and urinary transferrin level by Pearson's correlation test. In summary, LP treatment caused significant reduction in total urinary protein excretion and changes in urinary protein compositions that could be linked to stone inhibitory effects and might be relevant mechanisms responsible for the beneficial effects of LP to prevent kidney stone formation and recurrence.

Keywords Calcium oxalate · Kidney stone · Proteome · Proteomics · THP · Urinary proteins

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Introduction

Urolithiasis [the presence of stone(s) along urinary tract] is a common urological disease worldwide. The lifetime prevalence of this disease is up to 12% in the United States [1] and even higher in some other areas (e.g., up to 17% in Thailand) [2]. Furthermore, the recurrence rate is approximately 50% after 10 years of the stone removal [3]. Formation of the stone within urinary system can cause ureteral obstruction and renal tubular cell damage induced by oxidative stress and inflammation [4, 5]. Our previous study has shown that urolithiatic patients manifested low-grade intrarenal inflammation and increased intrarenal mRNA expression levels of MCP-1 and IL-6 that were associated with impaired renal function [6]. In addition, urolithiasis is one of the

risk factors for chronic kidney disease (CKD) [7] and end-stage renal disease (ESRD) [8], as evidenced by the decrease of creatinine clearance and estimated glomerular filtration rate (eGFR) in the stone formers [9, 10].

The most common type of stones found in patients worldwide is calcium oxalate, of which aggravating factors include hypercalciuria, hyperoxaluria, and hypocitraturia. Among these, hypocitraturia is the most frequent risk factor found in >80% of calcium oxalate stone formers in Thailand [11]. Lime—a common citrus fruit in Southeast Asia—contains high amounts of citric acid and some antioxidants (e.g., polyphenol, flavonoids, and ascorbic acid) [11]. Interestingly, our previous study has found that lime powder (LP) exerted stone preventive properties by its citruric and alkalinizing effects [11]. However, whether LP also has any other beneficial effects to the stone formers remained unknown.

It is well known that some urinary proteins may play important roles in modulation of the stone formation. For example, nephrocalcin, trefoil factor-1 and Tamm–Horsfall protein (THP) have been demonstrated to serve as inhibitors for calcium oxalate crystal growth, whereas albumin and annexin II have an opposite activity and serve as the stone promoters [12, 13]. Boyce et al. [14] has previously reported that protein excretion in urolithiatic patients was increased 3–13 times when compared to the normal controls. Therefore, it may be possible that the stone preventive properties of LP are partly due to its effect on urinary protein excretion in the stone formers. Our present study thus aimed to address the effect of LP on excretory level and compositions of urinary proteins using a proteomics approach.

Materials and methods

Subjects

This study was performed in concordance to the guidelines detailed in Declaration of Helsinki and was approved by the ethical committee for research in human subjects in the fields of Thai traditional and alternative medicine, Ministry of Public Health, Thailand and by the institutional ethical committee for research in humans at Sappasit Prasong Hospital. The written informed consents were obtained from all the participants. From a total of 80 calcium oxalate stone formers recruited into our 2-year randomized clinical trial of LP effect, 10 patients with comparable age and clinical parameters were selected for this proteomic study to reduce potential biases for subsequent data interpretation.

Specimen collection and sample preparation

24-h urine specimens were collected from all subjects, at baseline (before) and after LP treatment for 6 months. The samples were preserved in thymol and frozen at -80°C until ready for protein precipitation by 75% ice-cold ethanol in an icebox for 30 min. The precipitants were collected by a low-speed centrifugation for 5 min and solubilized with SDT lysis buffer (4% SDS, 100 mM DTT, and 100 mM Tris-HCl; pH 7.6). Protein concentrations were measured using Bradford's method.

SDS-PAGE

Equal amount of proteins derived from the same group were pooled and a total of 10 μg proteins derived from each pooled sample were subjected to SDS-PAGE in a precast gel (12% separating with 4% stacking gels) using Mini-Protean III electrophoresis system (Bio-Rad Laboratories, Hercules, CA) at 220 V for 2 h. The separated proteins were then visualized by Coomassie Brilliant Blue staining.

Protein identification by nanoflow liquid chromatography coupled to tandem mass spectrometry (nanoLC–MS/MS)

Equal amount of proteins solubilized in SDT lysis buffer derived from the same group were pooled, reduced by heating at 95°C for 5 min, and then alkylated with 50 mM iodoacetamide at room temperature in the dark for 20 min. Thereafter, the proteins were then digested with sequencing-grade modified trypsin (Promega; Madison, WI) at a ratio of 1:50 (w/w) trypsin/protein at 37°C for 16–18 h. Thereafter, 5% formic acid in 80% acetonitrile was added to stop the enzymatic activity. The digested peptides were dried and finally resuspended in 0.1% formic acid prior to MS/MS analysis.

Peptide mixture was separated by two-column system consisted of the 3 cm long pre-column containing 5- μm C18 resin and the 15 cm long analytical column packed with 3- μm C18 resin. The 160-min gradient elution of 0.1% formic acid in acetonitrile was performed using nano-flow liquid chromatography (nano-LC system, Thermo Fisher Scientific; Waltham, MA). The eluted peptides were directly electrosprayed into the mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific). Each sample was made to run in technical replicates.

MS/MS data processing and protein quantitative analysis

The MS raw files were analyzed by integrated MaxQuant software suite (version 1.5.3.30) (<http://www.biochem>.

mpg.de/5111795/maxquant). Proteins were identified by searching against the UniprotKB/Swiss-Prot *Homo sapiens* database (downloaded on March 2016, containing 20,193 protein entries). The search parameters were set as follows: maximal number of missed cleavages = 1, minimum peptide length = 7 amino acids, fixed modification = cysteine carbamidomethylation, variable modification = methionine oxidation, mass deviation for the main search of precursor masses <4.5 ppm, and de novo mass tolerance for tandem mass (MS/MS) spectra <0.25 Da. The false discovery rate (FDR) was filtered at <0.01 against a decoy database. For quantitation, label-free quantification (LFQ) intensities were created and the output files were subsequently examined by Perseus software version 1.5.3.2 (<http://www.biochem.mpg.de/5111810/perseus>) or manually processed using Microsoft Excel.

Validation of the proteomic data

The LP-induced changes in levels of urinary proteins identified by quantitative proteomics analysis were confirmed by a conventional method. Transferrin and uromodulin (also known as Tamm–Horsfall protein or THP) were measured using corresponding ELISA kits (Mybiosource; CA, USA). For the correlation study, microalbumin level was analyzed by immunoturbidimetric assay, using Architect analyzer (Abbott).

Statistics

The data are reported as mean \pm SD. Comparisons between the two groups of samples were performed using Student's *t* test, whereas correlation between microalbuminuria and transferrin levels was analyzed by Pearson's correlation test. *p* values less than 0.05 were considered statistically significant.

Results

From a total of 10 stone formers, 5 were from the LP-treated group, whereas another 5 were from the placebo group. Their age, body mass index (BMI), gender, and several baseline clinical chemistry characteristics before treatment, including urine volume, pH, protein, calcium, citrate, magnesium, sodium, oxalate, phosphate, creatinine, and uric acid levels were comparable (Table 1).

With comparable level of total urinary protein before treatment (Table 1), we also monitored this laboratory parameter after treatment with LP or placebo for 6 months. The data showed that the total urinary protein level was markedly decreased after LP treatment, but remained unchanged in the placebo group (Fig. 1).

Table 1 Baseline characteristics of subjects in placebo vs. LP-treated groups

	LP group (<i>N</i> = 5)	Placebo group (<i>N</i> = 5)	<i>p</i> value
Age (years)	46.2 \pm 10.6	43.2 \pm 6.7	0.6073
Body mass index (BMI)	22.23 \pm 3.45	24.36 \pm 2.69	0.3082
Gender (M:F)	2:3	3:2	
Urine volume (L)	1.66 \pm 0.51	2.06 \pm 1.13	0.4877
Urine pH	7.11 \pm 1.25	6.35 \pm 0.40	0.2319
Urine protein (mg/g creatinine)	460.87 \pm 267.39	407.46 \pm 214.91	0.7367
Urine calcium (mg/day)	26.15 \pm 7.62	100.47 \pm 97.18	0.1266
Urine citrate (mg/day)	160.53 \pm 158.69	103.93 \pm 76.48	0.4928
Urine magnesium (mg/day)	44.96 \pm 31.98	33.59 \pm 12.62	0.4810
Urine sodium (mEq/day)	91.65 \pm 54.28	64.54 \pm 27.06	0.3650
Urine oxalate (mg/day)	32.48 \pm 16.32	44.37 \pm 27.89	0.4341
Urine phosphate (mg/day)	553.26 \pm 308.47	326.83 \pm 136.80	0.1719
Urine creatinine (g/day)	1.88 \pm 1.15	0.96 \pm 0.44	0.1322
Urine uric acid (mg/day)	651.44 \pm 443.09	485.70 \pm 146.73	0.4500

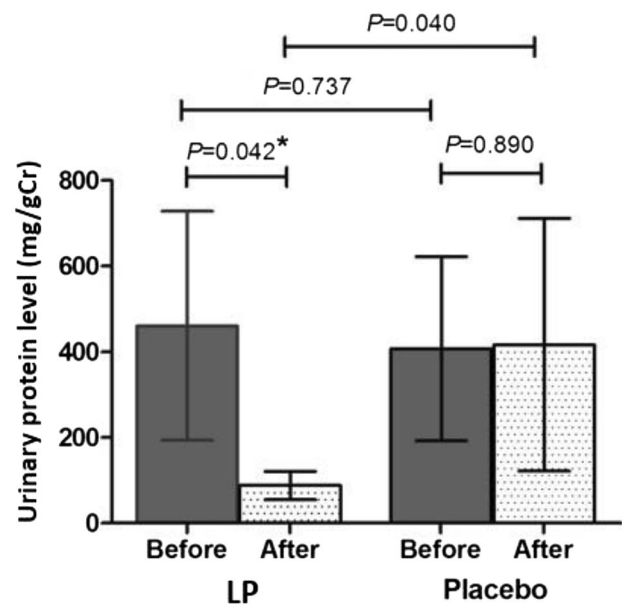


Fig. 1 Excretion of total urinary protein. The total urinary protein level within 24 h (mg of protein/g of creatinine) was compared before vs. after 6-month treatment by LP or placebo. The data are reported as mean \pm SD (*n* = 5/group)

SDS-PAGE showed obvious change of protein band pattern only in the LP group after the treatment (Fig. 2). These data indicated that there should be some obvious alterations in urinary proteome profile in the LP group after the treatment.

Quantitative proteomics analysis of the samples derived from LP (before vs. after treatment) and placebo (before vs. after treatment) groups revealed some common and unique changes by each treatment. There were 20 common proteins whose levels were significantly altered in both LP and placebo groups after 6-month treatment. Among these, 17 were decreased, whereas 3 were increased by both LP and placebo treatments (Table 2). These altered proteins might thus be the non-specific changes. Interestingly, there were the other 17 proteins whose levels were significantly altered (16 decreased and 1 increased) only by LP treatment (Table 3).

From these data, we selected two altered proteins for subsequent validation using conventional methods. For the unique changes induced by LP treatment, serotransferrin (or transferrin) was decreased with a fold-change of 0.09, whereas uromodulin (or Tamm–Horsfall protein; THP) was the only increased protein with a fold-change of 3.73. We thus measured urine transferrin and THP using corresponding commercial ELISA kits. The data showed that urinary excretion of transferrin tended

to decrease after 6-month LP treatment (with a marginal p value), but remained unchanged in the placebo group (Fig. 3a). Moreover, urinary THP excretion was significantly increased by the LP treatment, but no significant change was observed in the placebo group (Fig. 3b) consistent with the proteomic data.

Finally, we analyzed the correlation between microalbuminuria and transferrin levels in all the urine samples by Pearson's correlation test. The scatter plot showed a positive correlation between microalbumin and transferrin levels with a coefficient of correlation ($r = 0.589$ (p value = 0.006)) (Fig. 4).

Discussion

We have previously reported that LP treatment for 3 months decreased the stone-forming potential, improved antioxidative status, and reduced renal tubular damage in the stone formers [11, 15]. Moreover, LP could inhibit calcium oxalate crystal growth and provided citraturic, kaliuric, and antioxidative effects in healthy individuals without any adverse events [11, 15]. The present study further explored potential mechanisms underlying LP beneficial effects in the stone formers by demonstrating changes in urinary protein excretion and compositions using a proteomics approach, which has been demonstrated as the powerful tool in kidney stone research by several previous studies [16–19].

Protein is a major component of the stone matrix [20, 21] and is excreted with greater level in urolithiatic patients as compared to the healthy individuals [14]. We found that treatment with LP, not placebo, for 6 months significantly decreased total urinary protein excretion in urolithiatic patients (Fig. 1). The decreased level of urinary protein excretion is a good prognosis to avoid development of impaired renal function and chronic kidney disease (CKD), which are very common in urolithiatic patients [7]. Protein distribution pattern derived from SDS-PAGE presented several different bands in LP group of the urine samples (Fig. 2), indicating that not only the level, but also compositions of the urinary proteins were also altered by LP treatment.

Quantitative proteomics revealed a number of common proteins altered by both LP and placebo groups that could be considered as non-specific changes (Table 2). Most of these common decreased proteins were involved in immunity (Ig gamma/mu chains and complement factors C3/C4), inflammation (alpha-1-antitrypsin and lactotransferrin) and fibrotic process (fibrinogen beta/alpha/gamma chains), whereas the others were carrier proteins (albumin, haptoglobin, alpha-1-acid glycoprotein 1 and ceruloplasmin).

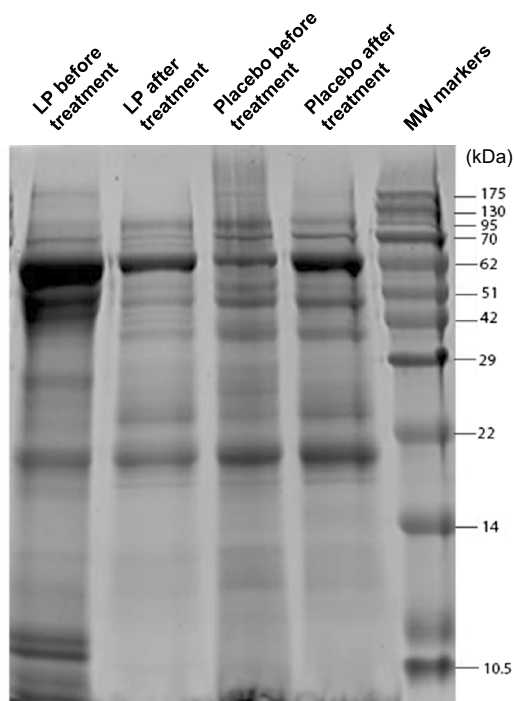


Fig. 2 SDS-PAGE. Proteins derived from each pooled sample with an equal amount (10 μ g/sample) were resolved by 12% SDS-PAGE and visualized by Coomassie brilliant blue staining

Table 2 Proteins with altered levels after treatment by both LP and placebo (sorted by %sequence coverage)

Accession no.	Protein name	MW (kDa)	Function	%Cov ^a		Fold-change ^b	
				LP	Placebo	LP	Placebo
Proteins whose levels were decreased after LP or placebo treatment							
P02768	Serum albumin	69.37	Plasma carrier protein	66.70	57.60	0.12	0.69
P01860	Ig gamma-3 chain C region	41.29	Immunity	39.50	32.40	0.05	0.23
P01861	Ig gamma-4 chain C region	35.94	Immunity	29.10	29.10	Absent	Absent
P00738	Haptoglobin	45.21	Plasma carrier protein	28.10	32.50	Absent	0.09
P02671	Fibrinogen alpha chain	69.76	Fibrosis	20.70	12.50	Absent	Absent
P01871	Ig mu chain C region	49.31	Immunity	19.90	15.70	Absent	Absent
P02679	Fibrinogen gamma chain	49.50	Fibrosis	19.20	26.50	Absent	Absent
P01024	Complement C3	187.15	Immunity	17.60	13.60	Absent	Absent
P01009	Alpha-1-antitrypsin	46.74	Inflammation	17.20	30.10	Absent	Absent
P02675	Fibrinogen beta chain	55.93	Fibrosis	16.30	15.30	Absent	Absent
P02788	Lactotransferrin	78.18	Immunity/inflammation	15.40	27.60	Absent	Absent
P01023	Alpha-2-macroglobulin	163.29	Coagulation	11.40	5.90	Absent	Absent
P02749	Beta-2-glycoprotein 1	38.30	Coagulation	10.40	11.30	Absent	Absent
P02763	Alpha-1-acid glycoprotein 1	23.51	Plasma carrier protein	9.00	12.90	Absent	Absent
P0C0L5	Complement C4-B	192.75	Immunity	7.60	5.30	Absent	0.32
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	93.85	Inflammation	6.70	5.00	Absent	Absent
P00450	Ceruloplasmin	122.20	Plasma carrier protein	5.60	4.80	Absent	Absent
Proteins whose levels were increased after LP or placebo treatment							
P02760	Protein AMBP	39.00	Trypsin inhibitor	44.30	44.30	3.48	1.82
P05090	Apolipoprotein D	21.28	Lipid metabolism	31.20	37.00	1.69	1.27
P25311	Zinc-alpha-2-glycoprotein	34.26	Lipid metabolism	13.10	27.90	2.17	3.75

“Absent” represents fold-change = 0 or protein level was undetectable after treatment

^a %Cov = %sequence coverage = (number of the matched residues/total number of residues in the entire sequence) × 100

^b Fold-change = mean of protein level after treatment/mean of protein level before treatment

There were 16 proteins that were decreased in levels exclusively after LP treatment (Table 3). These proteins were involved in various processes, such as immunity (Ig gamma/kappa/heavy chains and complement factor H), inflammation (peroxiredoxin-2 and alpha-1-antichymotrypsin), carrier proteins (serotransferrin and hemopexin), fibrosis or coagulation (fibronectin), and cellular proteins (hemoglobin subunits). In previous proteomic reports, inflammatory and fibrotic proteins were abundantly found in both urine and stone matrix from urolithiatic patients [12, 22, 23]. Their decreases might suggest the reduced immune, inflammatory and fibrotic processes by LP.

Among these unique changes after LP treatment, we selected transferrin and uromodulin as the representatives for LP-induced decreases and LP-induced increases, respectively, for subsequent validation. ELISA confirmed the decrease of transferrin and increase of THP levels,

exclusive after LP treatment, whereas there were no significant changes observed after placebo treatment (Fig. 3).

Transferrin (or serotransferrin, which can be used interchangeably) is an iron carrier protein in the plasma with a molecular mass of 76.5 kDa that also serves as a sensitive biomarker for glomerular damage. A previous study has demonstrated that transferrin could be found in both urine and stone matrix from urolithiatic patients [24]. In other disease models, transferrin excretion has been found to be correlated with microalbuminuria and other vascular complications in Type 2 diabetes and diabetic nephropathy [25]. We thus performed a correlation study of urinary transferrin and microalbuminuria in all the stone formers included in this study. The data revealed a positive correlation between urinary transferrin and microalbuminuria (Fig. 4), implicating that LP not only reduced protein excretion, but also improved kidney function as determined by the reduced

Table 3 Proteins with altered levels after treatment by LP only (sorted by %sequence coverage)

Accession no.	Protein name	MW (kDa)	Function	%Cov ^a	Fold-change ^b	<i>p</i> value
Proteins whose levels were decreased after LP treatment						
P68871	Hemoglobin subunit beta	16.00	Cellular protein	75.50	Absent	0.0048
P02787	Serotransferrin (transferrin)	77.06	Plasma carrier protein	40.80	0.09	0.0141
P01857	Ig gamma-1 chain C region	36.11	Immunity	39.70	Absent	0.0108
P02647	Apolipoprotein A-I	30.78	Lipid metabolism	28.10	Absent	0.0017
P01598	Ig kappa chain V-I region	11.79	Immunity	26.90	Absent	0.0060
P01765	Ig heavy chain V-III region	11.61	Immunity	26.10	Absent	0.0040
P32119	Peroxiredoxin-2	21.89	Immunity/inflammation	25.30	Absent	0.0178
P69905	Hemoglobin subunit alpha	15.26	Cellular protein	24.60	Absent	0.0225
P01859	Ig gamma-2 chain C region	35.90	Immunity	23.00	0.05	0.0193
P02774	Vitamin D-binding protein	52.96	Plasma/cellular carrier protein	22.40	Absent	0.0217
P01011	Alpha-1-antichymotrypsin	47.65	Inflammation	15.60	Absent	0.0495
P02652	Apolipoprotein A-II	11.18	Lipid metabolism	11.00	Absent	0.0479
P02790	Hemopexin	51.68	Plasma carrier protein	6.30	Absent	0.0345
P08603	Complement factor H	139.09	Immunity	4.10	Absent	0.0291
P02751	Fibronectin	221.29	Fibrosis/coagulation	3.00	Absent	0.0065
P04114	Apolipoprotein B-100	515.60	Lipid metabolism	1.10	Absent	0.0298
Proteins whose levels were increased after LP treatment						
P07911	Uromodulin	67.42	Urinary protein	21.70	3.73	0.0058

“Absent” represents fold-change = 0 or protein level was undetectable after treatment

^a %Cov = %sequence coverage = (number of the matched residues/total number of residues in the entire sequence) × 100

^b Fold-change = mean of protein level after treatment/mean of protein level before treatment

excretion of both total urinary protein (Fig. 1) as well as microalbuminuria (Fig. 4).

Interestingly, uromodulin (or THP) was the only one protein exclusively increased after LP treatment. THP is high abundant protein in the normal urine and its defects have been demonstrated to involve in many urological conditions, including tubular damage and urolithiasis [26, 27]. Many studies have reported that THP has a stone inhibitory effect [28, 29]. We observed the increase of THP only after LP treatment, consistent with the findings reported by Harold et al. [30] demonstrating the increased level of urinary THP in stone formers after potassium citrate treatment. Additionally, Ganter et al. [31] also found that urinary THP level was correlated with urinary citrate level in urolithiatic patients, and low excretion rate of THP might be due to tubular dysfunction. Citrate is a well known potent inhibitor for kidney stone formation and has an anti-inflammatory effect in endothelial cells and macrophages [32, 33]. Moreover, our previous studies have shown that urinary citrate level was increased after LP treatment in the stone formers

[11, 15]. Therefore, the increase of urinary THP is most likely another relevant beneficial effect of LP to prevent kidney stone formation or recurrence.

It should be noted that the number of subject included in this study was relatively small. Increasing such sample size would yield a more confident statistical power for defining significant changes as well as validation. Additionally, duration of LP treatment was limited to 6 months in the present study. Varying such duration or evaluation of spatial changes of the urinary proteome would enhance our understanding on the beneficial effects of LP against kidney stone formation. Nevertheless, our data report herein at least in part implicate that LP could prevent kidney stone formation via alterations in excretion of the stone modulators into the urine.

In summary, we reported herein a decrease of total urinary protein excretion and changes in excretion of 17 urinary proteins induced by LP treatment using a proteomics approach. Among these, the decrease of urinary transferrin and increase of THP were validated by ELISA. The changes of these two urinary proteins could be linked to

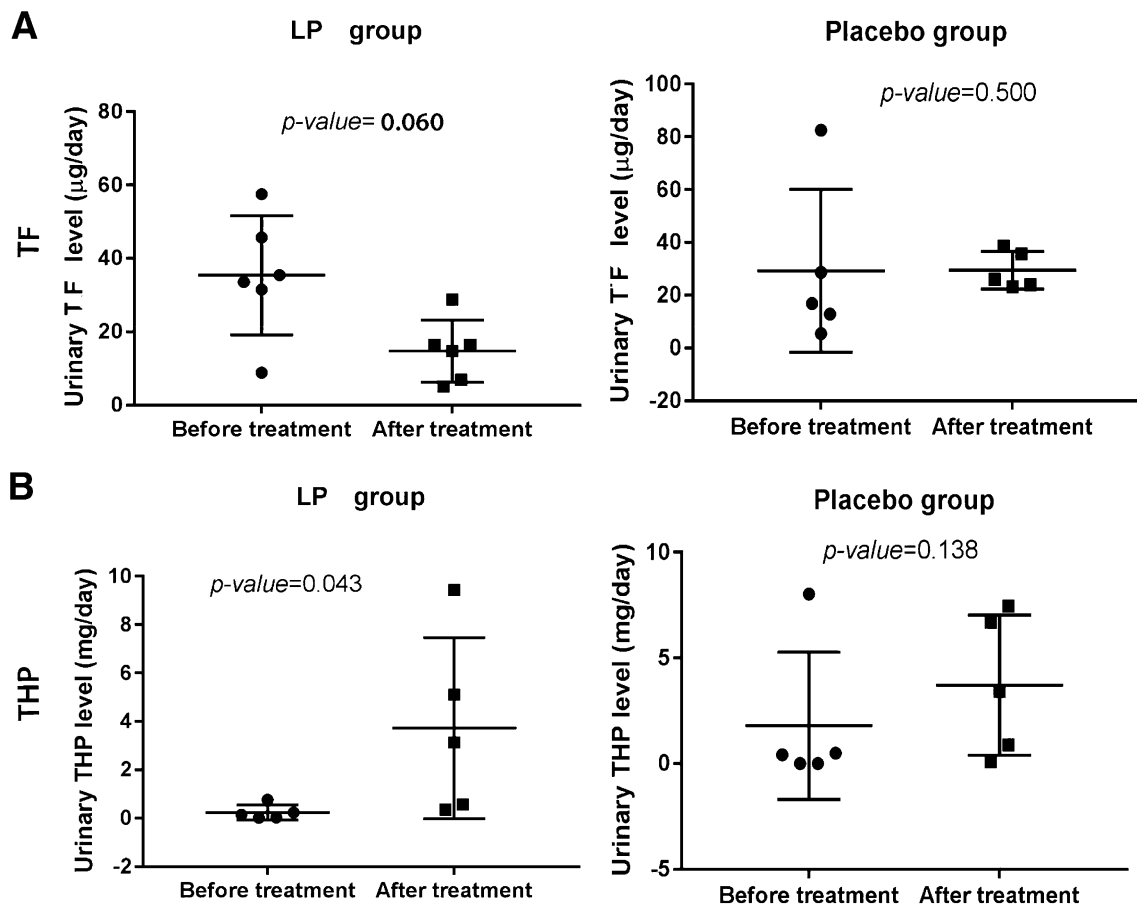


Fig. 3 Validation of the proteomic data by ELISA. **a** Urinary transferrin (TF) level before vs. after treatment with LP or placebo. **b** Uromodulin or THP level before vs. after treatment with LP or placebo. The data are reported as mean ± SD ($n = 5/\text{group}$)

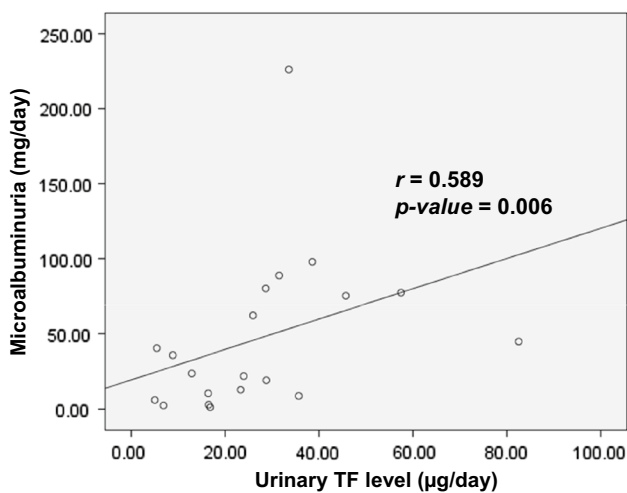


Fig. 4 Correlation between microalbuminuria and urinary transferrin (TF) level. The correlation was analyzed by Pearson’s correlation test ($n = 20$). r = Pearson’s correlation coefficient

stone inhibitory effects and may be relevant mechanisms responsible for the beneficial effects of LP to prevent kidney stone formation and recurrence.

Author contributions PT, PK, SC, WU, NK, SR and VT designed research; PK, SC, WU, NK and SR performed experiments; PT, PK, SC, WU, NK, SR and VT analyzed data; PT, PK and VT wrote the manuscript; All authors reviewed and approved the manuscript.

Compliance with ethical standards

Funding This study was supported by National Research Council of Thailand, Mahidol University research grant, Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, and the Thailand Research Fund (RTA5680004 and IRG5980006). SC and VT are also supported by Faculty of Medicine Siriraj Hospital.

Conflict of interest All Authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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