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ORIGINAL PAPER

Lithogenic activity and clinical relevance of lipids extracted from urines and stones of nephrolithiasis patients

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Abstract We investigated contents and classes of urinary and stone matrix lipids, and evaluated their clinical relevance in nephrolithiasis patients. Lithogenic role of major lipid classes was explored. Urine (24 h) and stone samples were collected from 47 patients with nephrolithiasis. Control urines were obtained from 29 healthy subjects. Urinary 8-hydroxy-deoxyguanosine (8-OHdG), malondialdehyde (MDA), *N*-acetyl-β-glucosaminidase (NAG) activity and total proteins were measured. Total lipids were extracted from centrifuged urines (10,000 rpm, 30 min) and stones by chloroform/methanol method. Major classes of lipids were identified using multi-one-dimensional thin-layer chromatography (MOD-TLC). Influence of each lipid class purified from stone matrices on stone formation was evaluated using crystallization and crystal aggregation assays. Urinary NAG activity and 8-OHdG were significantly elevated in nephrolithiasis patients. Total lipids in centrifuged urines of the patients were not significantly different from that of controls. In nephrolithiasis, urinary excretion of total lipids was linearly correlated to urinary MDA, 8-OHdG, NAG activity and total proteins. Lipid contents in stone matrices varied among stone types. Uric acid stone contained lower amount of total lipids than calcium oxalate and magnesium ammonium phosphate stones. MOD-TLC lipid chromatograms of healthy urines, nephrolithiasis urines and stone matrices were obviously different. Triacylglyceride was abundant in urines, but scarcely found in stone matrices. Stone matrices were rich in glycolipids and high-polar lipids (phospholipids/gangliosides). Partially purified glycolipids significantly induced crystal aggregation while cholesterol was a significant inducer of both crystal formation and agglomeration. In conclusion, total lipids in centrifuged urines did not differ between nephrolithiasis and healthy subjects. Our finding suggests that the significant sources of lipids in patients' urine may be large lipids-containing particles, which are removed in centrifuged urines. However, urinary lipid excretion in nephrolithiasis patients was associated with the extent of oxidative stress and renal tubular injury. Triacylglyceride was abundant in urines, but rarely incorporated into stones. Glycolipids were principal lipid constituents in stone matrices and functioned as crystal aggregator. Cholesterol purified from stone matrices bared crystal nucleating and aggregating activities.

Keywords Nephrolithiasis · Urinary lipids · Stone matrix lipids · Crystal aggregation · Crystallization · Glycolipids

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Introduction

Kidney stone disease is endemic in Thailand, and it is one of serious health problems in rural communities of the

country [1]. Most patients are inflicted with large stones which frequently recur. Due to large number of patients, their low economic status and limited number of fully equipped hospitals, the patients have to wait for 4–6 months to receive their surgical operation. Although the disease is not a direct cause of death, large stone formation and multiple recurrences straightforwardly lead to end stage renal disease [2]. A large fraction of nephrolithiasis patients in Thailand, particularly those with staghorn stones, are subsequently inflicted with chronic renal failure. Most of them face financial problem for extensive dialysis and renal transplantation and refuse the therapy—they shortly die after that (personal communication).

It is well known that urinary crystals are building blocks of kidney stones, but how microscopic crystals transform to macroscopic stones is not fully understood. However, stones are not solely made of crystals. A variety of organic materials such as whole cells, cell debris, membrane vesicles and cellular macromolecules are always found in stones, so call organic matrix. It is believed that organic matrix acts as biological cement to build stones from the crystals [3]. On the other side, various evidences suggest that cellular biomolecules in urine, which are actively and selectively incorporated into crystals and stones, provide physiological function in preventing stone development via enhancing degradation of phagocytosed crystals in renal epithelial cells as well as inhibiting crystal formation [4–7]. Lipid is one of the cellular biomolecules that is frequently found in stone matrix, and has been suggested to play a significant role in lithogenic process. Lipid matrix in the form of cellular membrane is demonstrated to act as nidi of calcium apatite nucleation [8]. Histological examination reveals sudanophilic substances in sections of demineralized calcium oxalate (CaOx) stone, indicating a presence of lipids [9, 10]. Lipid matrix extracted from EDTA-insoluble matrix is capable of nucleating CaOx crystals in metastable solution [11]. Rat renal membrane vesicles are shown to induce CaOx crystallization in vitro [12, 13]. Increase in urinary excretion of lipids (whole urine) is documented in kidney stone patients [14]. Khan et al. [15] demonstrated that patients with uric acid (UA) stone ($n = 3$) excreted urinary proteins, glycolipids, cholesterol, cholesterol ester and triglyceride greater than healthy controls ($n = 8$), whereas CaOx stone formers excreted urinary proteins, cholesterol, cholesterol ester, triglyceride and phospholipids more than the controls ($n = 12$). They also showed that average lipid contents in CaOx ($n = 5$) and calcium phosphate ($n = 3$) stones were apparently higher than that in UA ($n = 5$) and struvite ($n = 5$) stones. Although their study provides significant findings, number of samples is relatively small. Additional study is required to corroborate the concluded figures.

In this study, total lipid contents in urine and stone samples obtained from nephrolithiasis patients were determined. Their associations with clinical characteristics were also assessed. Instead of whole urine, we extracted lipids from centrifuged urines asked if solubilized/suspended urinary lipids was increased in nephrolithiasis patients. In addition, major classes of lipids in stone matrix and their role in crystallization and crystal aggregation were investigated.

Methods

Subjects and specimen collection

A total of 76 subjects divided into nephrolithiasis patients ($n = 47$) and healthy volunteers ($n = 29$) who had no history of stone formation (evaluated by direct interview) were recruited (Table 1). Nephrolithiasis patients aged 18–72 (47 ± 14) years consisted of 21 (45%) males and 26 (55%) females. Healthy subjects aged 20–58 (39 ± 10) years consisted of 8 (28%) males and 21 (72%) females. The patients admitted at Rajavithi Hospital and King Chulalongkorn Memorial Hospital, Bangkok underwent surgical management for stone removal by either open stone surgery or percutaneous nephrolithotomy or extracorporeal shockwave lithotripsy. Written informed consents were received from all participants, and the research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Urine specimens (24 h) were collected from all subjects, using thymol as preservative. In patient group, pre-operative urine samples were collected, and kept at –20°C until testing. Any specimens with a urinary creatinine (Cr) level of <0.5 g/day were regarded as an inadequate collection and excluded or re-collected.

Table 1 Oxidative stress, renal tubular damage and urinary lipid excretion compared between nephrolithiasis and healthy controls

Urinary parameters	Healthy	Nephrolithiasis	*P* value
n	29	47	
Males:females	8:21	21:26	
Age (years)	39.38 ± 10.21	47.36 ± 14.11	
BMI (kg/m^2)	21.76 (3.13)	23.46 (5.49)	0.014
Volume (ml)	$2{,}207 \pm 776$	$1{,}652 \pm 739$	0.003
pH	6.40 (0.28)	6.16 (0.94)	0.044
Creatinine (g/day)	0.80 (0.58)	0.78 (0.36)	0.104
NAG activity (U/g Cr)	3.90 (3.53)	8.30 (9.69)	<0.001
8-OHdG (μg/g Cr)	5.37 (4.49)	7.61 (6.99)	0.003
Lipids (mg/g Cr)	550.7 (682.2)	783.3 (525.8)	0.122
Lipids (mg/day)	626.7 (655.6)	561.9 (328.3)	0.979

Data presented as mean ± SD or median (IQR)

Urinary *N*-acetyl-β-glucosaminidase (NAG) activity and proteins, indicators of renal tubular injury, were measured using spectrophotometric and dye-binding methods, respectively. Urinary 8-hydroxy-deoxyguanosine (8-OHdG), a biomarker of oxidative stress, was determined by competitive ELISA (New 8-OHdG Check ELISA kit, JaICA, Japan). Urinary malondialdehyde (MDA), a marker of lipid peroxidation, was measured by thiobarbituric acid assay.

Stone specimens obtained from nephrolithiasis patients were thoroughly washed three times with distilled water, dried at 60°C overnight, and pulverized. Mineral composition of pulverized stone was analyzed by FTIR spectroscopy. Stone types were categorized into calcium oxalate (CaOx, $n = 29$), magnesium ammonium phosphate (MAP, $n = 7$) and uric acid (UA, $n = 11$) stones. Demographic characteristics and urinary metabolic profile of stone formers is shown in Table 2. There were no significant differences of urinary parameters among the three groups.

Lipids extraction from centrifuged urines and stones

Total lipids were extracted from centrifuged 24-h urine samples by chloroform/methanol technique. In brief, urine sample was centrifuged at 10,000 rpm, 15°C for 30 min, and the sediment was discarded. The centrifuged urine (1 part) was added with chloroform/methanol (2:1, v/v) (3 parts). The mixture was continuously mixed by orbital mixer overnight (16 h). Organic phase (lower phase) as total lipid extract was collected using separating funnel. The total lipid extract was evaporated until dryness using Speedvac concentrator and weighted. Dried lipid extract was re-dissolved in chloroform/methanol containing 0.1% butyl hydroxytoluene (BHT) and kept at −20°C for further analysis.

To extract total lipids from stone materials, 2 g of stone powder was re-suspended with 40 ml of an extraction solvent, ice-cold chloroform:methanol:0.05 M Tris–HCl, pH 7.4 (2:1:1). The mixture was sonicated for 20 min, continuously mixed by orbital mixer for 16 h and centrifuged at 10,000 rpm for 10 min. The organic phase was collected as lipid extract I. Stone pellet was further extracted with extraction solvent for 2 h, and the organic phase was collected as lipid extract II. The remained stone pellet was re-extracted for 2 h, and lipid extract III was collected. The lipid extract I, II and III were pooled (as total stone matrix lipids), evaporated, weighted, re-dissolved with chloroform/methanol containing 0.1% BHT and kept at −20°C for further analysis.

Multi-one-dimensional thin-layer chromatography (MOD-TLC)

The MOD-TLC, a multistep TLC technique, was employed to separate major lipid classes in the total lipid extracts [16]. Total lipid extracts were spotted onto high performance TLC (HPTLC) plate (Sigma-Aldrich, St. Louis, USA). After spots were completely dried, MOD-TLC was run for 2 h in equilibrated TLC chamber as follows. First, the plate was run to 2.5 cm from the bottom (approximately 2 min) in chloroform:methanol:acetic acid (90:10:1, v/v/v). The plate was then dried with a hair dryer, and run in hexane:diethyl ether:acetone (60:40:5, v/v/v) to 8.0 cm from the bottom, over approximately 10 min. The plate was dried again, and run in the third solvent, hexane:diethyl ether (97:3, v/v), to 9.5 cm (approximately 15 min). At final step, the dried plate was run in 100% hexane to 10 cm (approximately 30 min). Lipid spots were visualized by exposure to iodine vapor, and their retention factors (R_f) were calculated. Lipid classes were identified by comparing their R_f to those of known standards. Mobility of each lipid classes on HPTLC stationary phase is inversely related to its polarity, meaning that higher polar lipids move slower (lower R_f values) than the lower polar lipids.

Table 2 Demographic characteristics and urinary metabolic profile of the studied patients

Variables	Kidney stone formers			*P* value
	CaOx	MAP	UA	
n	29	7	11	
Males:females	11:18	3:4	7:4	
Age (years)	45.86 ± 12.61	46.71 ± 17.43	51.73 ± 16.13	0.297
BMI (kg/m^2)	22.94 (4.31)	24.89 (9.78)	24.97 (5.43)	0.383
U-calcium (mg/day)	62.16 (66.12)	20.72 (27.44)	44.59 (57.57)	0.081
U-phosphate (g/day)	0.36 (0.25)	0.40 (0.10)	0.44 (0.74)	0.865
U-oxalate (mmol/day)	0.15 (0.55)	0.17 (0.16)	0.10 (0.24)	0.376
U-uric acid (mg/day)	473.08 (218.86)	446.97 (351.08)	330.12 (305.59)	0.414
U-potassium (mEq/day)	15.75 (12.91)	20.13 (22.75)	20.30 (25.76)	0.611
U-citrate (mg/day)	60.79 (65.01)	59.04 (106.99)	26.29 (71.06)	0.513

Data presented as mean ± SD or median (IQR)

U urine, *mEq* milliequivalent

To visualize glycolipids, periodic acid Schiff (PAS) staining was performed on the developed HPTLC plate [17]. In brief, the developed HPTLC plate was washed with distilled water for 2 × 10 min. The plate was immersed in oxidizing solution [0.02% (w/v) periodic acid/0.09% (v/v) acetic acid] for 30 min. The oxidized plate was washed with 0.1% (w/v) sodium metabisulfite in 1 M HCl for 2 × 5 min, and then incubated in Schiff's Fuchsin-sulfite reagent (Sigma-Aldrich, St. Louis, USA) for 15 min. The plate was washed with sodium metabisulfite solution for 1 × 5 min, and dried with a hair dryer.

In vitro crystallization assay

CaOx crystallization assay was carried out in pooled healthy urine as described earlier [18]. Pooled healthy urine was centrifuged at 10,000 rpm for 10 min. The supernatant (2 ml) was placed into test tubes and warmed to 37°C for 10 min. Total lipids (from healthy urine, nephrolithiasis urine and stone matrix) at various amounts (50, 100 and 150 μg) was added and thoroughly mixed. Each tube was added with 50 μl of 0.1 M sodium oxalate, mixed and incubated at 37°C for 30 min. Number of crystals formed in each tube was counted under light microscope using hemacytometer.

To assess the effect of lipid classes on CaOx crystal formation, stone matrix total lipids were chromatographed on HPTLC plates to separate lipid classes. Silica gel containing each lipid class was scraped from the developed HPTLC plates and pooled. Each lipid class was extracted from silica gel by chloroform/methanol, then dried and weighted. Amount of 200 μg was used for crystallization assay as described above. All experiments were done in triplicate.

In vitro aggregation assay

Seed calcium oxalate monohydrate (COM) was prepared by mixing equal volume of 100 mM calcium chloride and 100 mM sodium oxalate. The mixture was sequentially incubated at 60°C for 1 h, and 37°C overnight, and then filtered through 0.22 μm membrane for collecting COM crystals. The seed crystals were dried at 37°C. Working solution of seed COM crystals was prepared (0.5 mg/ml of seed COM crystals in 0.05 M Tris/0.15 M sodium chloride, pH 6.5).

To assess aggregating activity of total lipid extract, 200 μg of lipid extract was placed into test tube, and 2 ml of working COM solution was added. After mixing, baseline absorbance at 620 nm was measured (AT_0). The mixture was then incubated at 37°C for 10 min, and the second absorbance was recorded (AT_{10}). Increased crystal aggregation is corresponded with decreased absorbance. The aggregation coefficient (AC) was calculated as follows: $AC = [(AT_0 - AT_{10})/10] \times 1{,}000$ [19]. A higher crystal aggregation is indicated by a higher AC value. Aggregated crystals were confirmed under light microscope. AC value of each lipid class purified from stone matrices was also determined. All experiments were performed in triplicate.

Statistical analysis

Data presented as the mean ± SD or median (interquartile range, IQR) as appropriated. Two independent groups were compared by a two-sample *t* test or Mann–Whitney *U* test where appropriate. Differences among three independent groups were assessed by a Kruskal–Wallis test, and Bonferroni correction was used as multiple comparison test. Association between two variables was evaluated by Spearman's rank correlation test. All tests were carried out using STATA version 8 (College station, TX, USA). Scatter plots were created by SPSS version 13 (Chicago, IL, USA). A two-sided $P < 0.05$ was used to indicate significance.

Results

Forty-seven patients with nephrolithiasis aged 18–72 (47.36 ± 14.11) years and 29 healthy controls aged 20–58 (39.38 ± 10.21) years were recruited (Table 1). The patient group consisted of 21 men (44.68%) and 26 women (55.32%), and control group contained 8 men (27.59%) and 21 women (72.41%). BMI of nephrolithiasis patients was significantly greater than that of the controls [median (IQR) 23.46 (5.49) vs. 21.76 (3.13) kg/m^2] ($P = 0.014$).

Patients with nephrolithiasis had significantly lower urine volume and pH than the controls (Table 1). Urinary levels of NAG activity and 8-OHdG in patient group were significantly higher than in control group (Table 1). Oxidative stress and renal tubular damage among patients with different stone types were also explored. Although significant differences of oxidative stress and renal tubular damage extents among patients of different stone types were not observed, patients with MAP stone [urinary 8-OHdG 9.94 (16.09) μg/g Cr, NAG activity 10.12 (25.07) U/g Cr] trended to have higher degrees of oxidative stress and renal tubular damage than those with CaOx [urinary 8-OHdG 7.99 (5.67) μg/g Cr, NAG activity 8.69 (10.17) U/g Cr] and UA [urinary 8-OHdG 7.61 (6.99) μg/g Cr, NAG activity 8.30 (9.69) U/g Cr] stones.

Levels of total lipids in centrifuged urines between nephrolithiasis patients and controls were not significantly different neither expressed as mg/g Cr nor mg/day ($P > 0.05$) (Table 1). Likewise, among patients with CaOx [median (IQR) 808.33 (546.69) mg/g Cr], MAP

[median (IQR) 767.70 (243.68) mg/g Cr] and UA [median (IQR) 831.46 (504.73) mg/g Cr] stones, total lipid contents in their centrifuged urines were not significantly different ($P = 0.576$). As well, there were no significant difference in urinary total lipids excretion between men and women, both in patient and control groups.

Urinary total lipids was significantly correlated to urinary MDA (Spearman's rho 0.501, $P < 0.001$; Fig. 1a), 8-OHdG (Spearman's rho 0.400, $P = 0.005$; Fig. 1b), NAG activity (Spearman's rho 0.342, $P = 0.019$; Fig. 1c) and total proteins (Spearman's rho 0.387, $P = 0.007$; Fig. 1d). This finding suggested that increased urinary level of total lipids was associated with increases in oxidative stress and renal tubular injury.

Of 47 patients, 36 had enough stone materials for lipid extraction classified as CaOx ($n = 23$, 63.89%), UA ($n = 7$, 19.44%) and MAP ($n = 6$, 16.67%). Contents of stone matrix lipids compared among the three stone types were significantly different ($P = 0.044$) (Fig. 2). UA stone [median (IQR) 1.0 (1.1) mg/g stone] contained total lipids significantly lower than CaOx [median (IQR) 1.8 (1.2) mg/g stone] and MAP [median (IQR) 2.3 (1.8) mg/g stone] stones.

To separate and identify major classes of urinary and stone matrix lipids, MOD-TLC was performed. At least 11

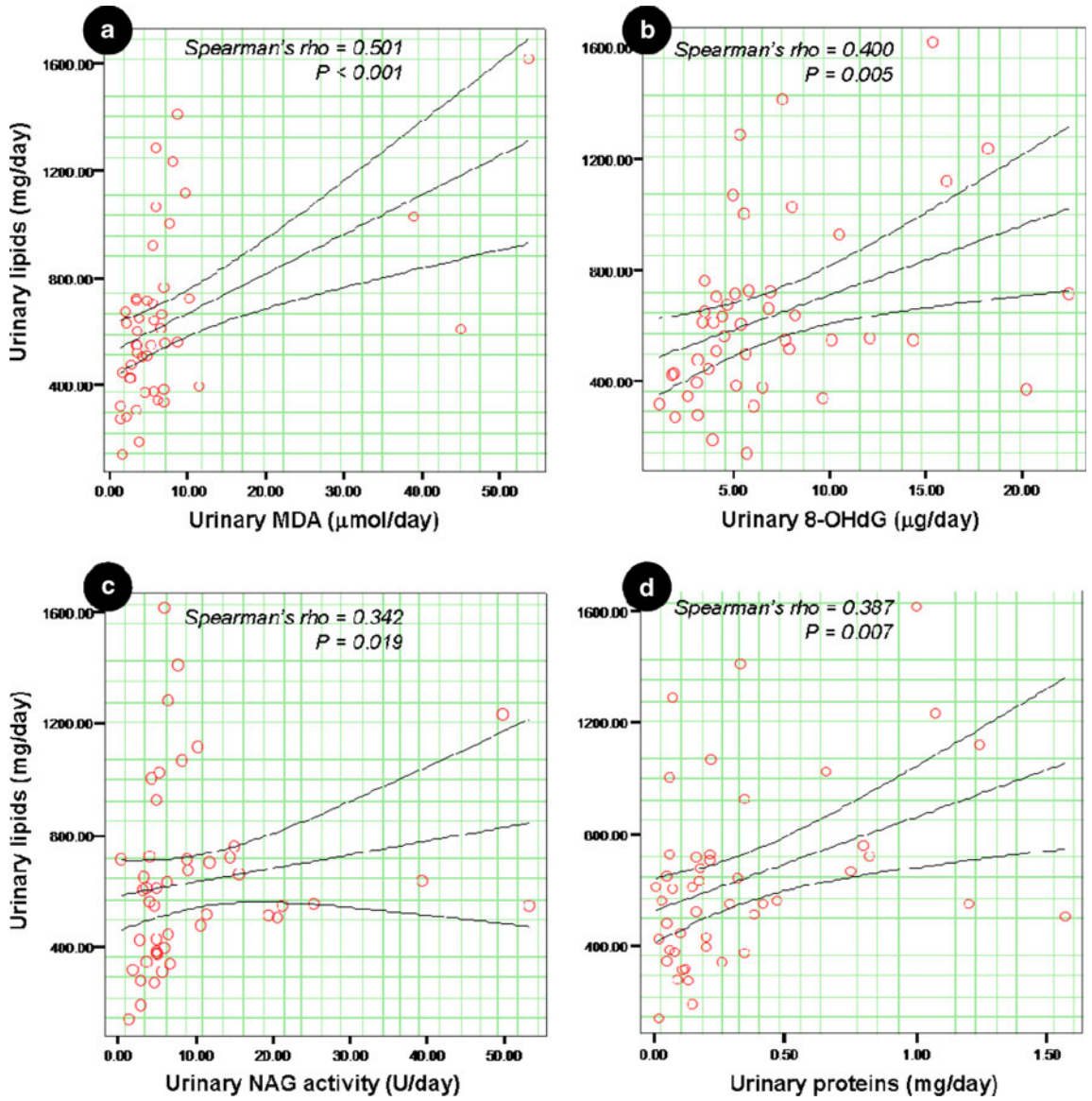

Fig. 1 Scatter plots and bivariate correlation between urinary total lipids and urinary excretions of MDA, 8-OHdG, NAG activity and total proteins in nephrolithiasis patients. Urinary level of total lipids was positively correlated with urinary MDA, 8-OHdG, NAG activity and total proteins with Spearman's rho of 0.501, 0.400, 0.342 and 0.387 ($P < 0.05$ for all), respectively

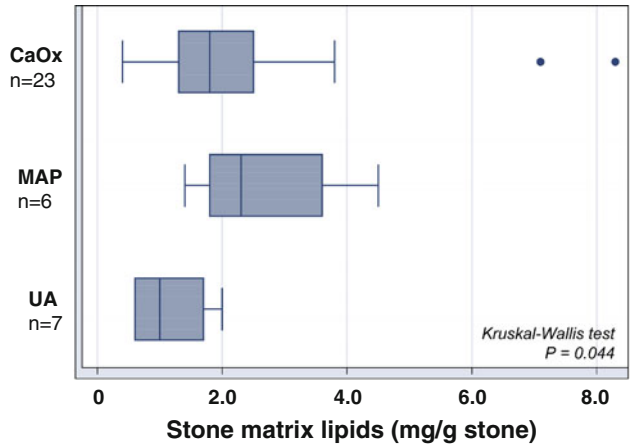

Fig. 2 Box-Whisker plot of total lipid contents in each type of kidney stones. Stone matrix lipids compared among CaOx, UA and MAP stones were significantly different ($P = 0.044$). UA stone contained total lipids considerably lower than CaOx and MAP stones

lipid spots (B1–B11) were found according to iodine and PAS stainings (Fig. 3). Of 11 spots, five lipid classes were successfully identified in accordance with R_f of known standards. B6, B7, B8 and B11 were identified as cholesterol, free fatty acids, triacylglyceride and cholesterol esters, respectively. Phospholipids and gangliosides standards are highly charged and revealed no movement on MOD-TLC system ($R_f = 0$). Therefore, we defined B1 as phospholipids/gangliosides (Fig. 3). Due to a lack of standards, six spots (B2, B3, B4, B5, B9 and B10) were tagged as unknown. However, all unknown spots were strongly positive for PAS staining indicated that these lipids were sugar-containing lipids or glycolipids.

The MOD-TLC chromatograms showed that lipid classes in healthy urines, nephrolithiasis urines and stone matrices were notably different (Fig. 4). All 11 lipid spots were found in stone matrices, but only few lipid spots were observed in urine samples. In healthy urines, only B1 (phospholipids/gangliosides), B5 (glycolipids), B8 (triacylglyceride) and B11 (cholesterol esters) were found. In addition to B1, B5, B8 and B11, B6 (cholesterol) and B9 (low-charged glycolipids) were found in nephrolithiasis urines. PAS-positive lipids were prevalent in stone matrices, and these sugar-containing lipids were more frequently detected in patients' urine than in controls' urine. Interestingly, triacylglyceride was the most abundant lipid found in urine samples (both healthy and nephrolithiasis), but it was almost absent in the stone matrices. Free fatty acids were found in all stone samples, but they were undetectable in urine samples. Compared among stone types, MOD-TLC chromatograms of stone matrix lipids were relatively similar (Fig. 4). Moreover, urinary lipid chromatograms of patients with different stone types were not obviously different.

Crystal formation induced by urinary and stone matrix total lipids was determined using in vitro crystallization assay. Total lipids from all sources induced

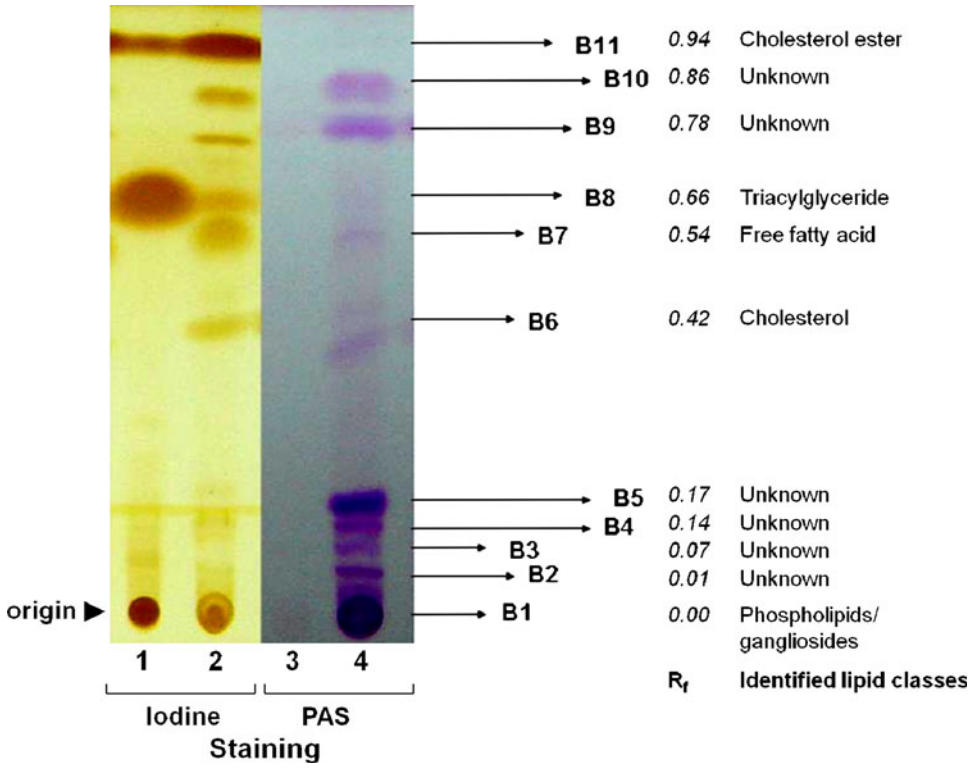

Fig. 3 MOD-TLC chromatogram shows all lipid spots observed in urine and stone samples. *Lanes 1* and *3* were urinary total lipids, while *lanes 2* and *4* were stone matrix lipids. Based on iodine (*lanes 1–2*) and PAS (*lanes 3–4*) stainings, 11 lipid spots (*B1–B11*) were found. Five spots were successively identified regarded to R_f of lipid standards viz. phospholipids/gangliosides (*B1*), cholesterol (*B6*), free fatty acids (*B7*), triacylglyceride (*B8*) and cholesterol ester (*B11*). Six PAS-positive spots (*B2–B5* and *B9–B10*) remained unidentified. R_f retention factor

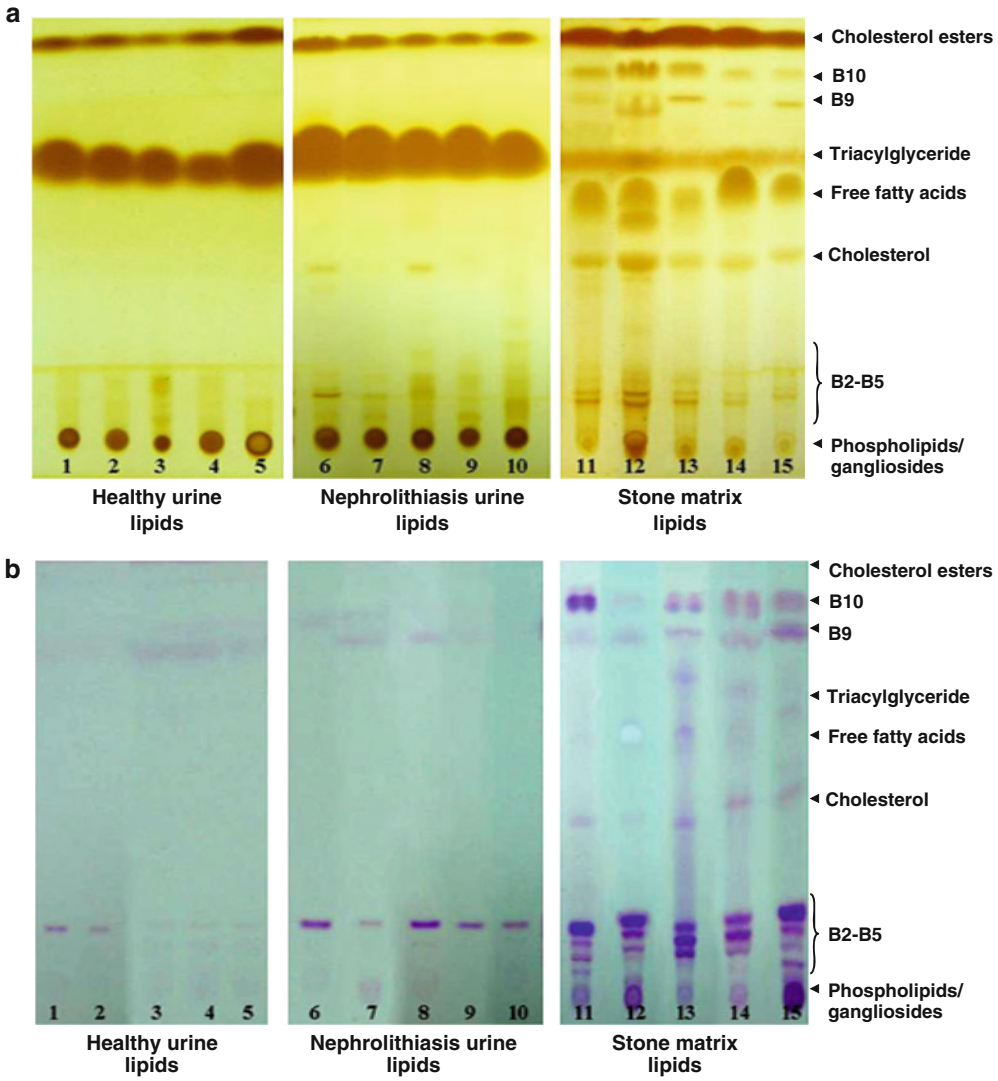

Fig. 4 MOD-TLC chromatograms show lipid classes in five different samples of healthy urines (*lanes 1–5*), nephrolithiasis urine (*lanes 6–10*) and stone matrices (*lanes 11–15*). Iodine (**a**) and PAS (**b**) stainings revealed that lipid patterns among the three sources were obviously different. In stone matrices, all 11 lipid classes were detected. Only a few lipids classes were presented in urine samples. PAS-positive lipids (*B2–B5* and *B9–B10*) were frequently detected in stone matrices, but rarely seen in healthy urines. More lipid classes and PAS-positive lipids were detected in nephrolithiasis urines than the healthy urines. Triacylglyceride was abundant in both healthy and nephrolithiasis urines, but it was scarce in stone matrices. Free fatty acids and cholesterol were found in all stone samples, but they were hardly found in urines. Stone matrix lipid chromatograms compared among stone types were relatively similar (UA: *lanes 11* and *13*, CaOx: *lanes 12* and *14*, MAP: *lane 15*)

crystallization in dose-dependent manner (Fig. 5). Notably, total lipids from stone matrix were capable of inducing crystal formation higher than total lipids from nephrolithiasis and healthy urines (Fig. 5). At 100 μg of total lipids, crystal-forming activity of stone matrix lipid was significantly greater than that of urinary lipids (Fig. 5). As expected, crystal-forming activity of total lipids from patients' urines was greater than that from controls' urines.

Crystal-forming activity of each lipid class purified from stone matrix was determined. High- and low-polar PAS-positive lipids (Fig. 3) were pooled and named as Pooled B2-B5 and Pooled B9-B10, respectively. Table 3 displays crystal numbers induced by each lipid class from stone matrix. A significant increase in crystal numbers compared to control was found only for cholesterol (B6).

In aggregation assay, stone matrix total lipids efficiently induced COM agglomeration. AC value of stone matrix total lipids was significantly greater than those of urinary total lipids and control (Fig. 6A). Micrographs of crystal aggregates are shown in Fig. 6B, which are corresponded well with AC values. The AC values of healthy and nephrolithiasis urinary total lipids were not statistically different (Fig. 6A).

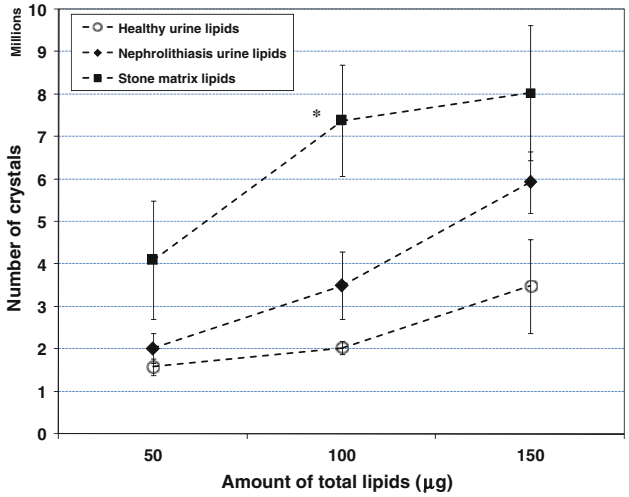

Fig. 5 Effect of total lipids isolated from healthy urines, nephrolithiasis urines and stone matrices on crystal formation. Total lipids from all sources induced crystal formation in dose-dependent fashion. At 100 µg of total lipids, crystal numbers induced by stone matrix lipids was significantly higher than those induced by urinary lipids. In addition, crystal induction ability of urinary total lipids from nephrolithiasis patients was higher than that of urinary total lipids from healthy controls. *Error bars* represent standard errors

Table 3 Effect of each lipid class isolated from stone matrices on crystal formation

Purified lipids	Crystal number (crystals/ml)	*P* value
Control	315,000 ± 140,000	
B1 (phospholipids/ gangliosides)	445,000 ± 515,437	1.000
Pooled B2–B5	375,000 ± 422,411	1.000
B6 (cholesterol)	7,643,333 ± 1,985,613	<0.001
B7 (free fatty acids)	60,000 ± 13,229	1.000
B8 (triacylglyceride)	205,000 ± 177,975	1.000
Pooled B9–B10	118,333 ± 41,633	1.000
B11 (cholesterol esters)	120,000 ± 27,839	1.000

Data presented as mean ± SD. *P* from Bonferroni multiple comparison test versus control

Influence of each lipid class isolated from stone matrix on crystal aggregation was investigated. All lipid classes had AC values higher than control (Table 4). However, only Pooled B2-B5, cholesterol and Pooled B9-B10 had AC values significantly greater than the control. AC values of phospholipids/gangliosides (B1) and free fatty acids (B7) were also increased but marginally significantly (both $P = 0.055$) (Table 3). The neutral lipids, triacylglyceride (B8) and cholesterol esters (B11) had the least effect on COM aggregation.

In selected cases ($n = 3$ each group), we examine the lipid constituent of urine sediments and found that nephrolithiasis urine sediments contained more debris and cellular fragments than the sediments from healthy urines (Fig. 7a, b). Lipid content in urine sediment of nephrolithiasis patients was higher than that in urine sediment of healthy controls (8.00 ± 4.88 vs. 3.27 ± 1.55 mg/30 ml urine). Moreover, MOD-TLC chromatograms revealed more glycolipids in nephrolithiasis urine sediments compared to healthy urine sediments (Fig. 7c).

Discussion

Lipiduria is well recognized and characterized in nephrotic syndrome, and has also been reported in nephrolithiasis [14, 15]. We herein investigated the quantity and quality of lipids in nephrolithiatic urines and stone matrices as well as explored their roles in lithogenic process. Our findings clearly show that stone matrices contained several classes of lipids. Glycolipids and cholesterol in particular play a significant role in lithogenesis as indicated by crystallization aggregation assays. In addition, level of urinary lipids excretion was associated with oxidative stress and renal tubular injury in nephrolithiasis patients.

Khan and Glenton [14] first reported that kidney stone formers ($n = 10$) excreted urinary lipids (whole urine) higher than normal individuals ($n = 6$). They also showed that urine sediment examined by TEM contained membrane fragments and exfoliated cells, which were suggested as a source of urinary total lipids. In the present study, we used centrifuged urines asked if the content of solubilized or suspended lipids in urines of nephrolithiasis patients was also higher than that in healthy urines. We did not find a significant difference of total lipids in centrifuged urine between patient and healthy groups. However, our preliminary data (Fig. 7) showed that lipid content in urine sediment of nephrolithiasis patients was higher than that in urine sediment of healthy controls. Another study of Khan et al. [13] demonstrated that human urine contained cellular membranes, and lipid content in the urine was significantly reduced after centrifugation and ultrafiltration. We believe that the exfoliated cells, membrane fragments/vesicles and other forms of large lipids-containing particles are significant sources of urinary lipids in nephrolithiasis patients. We should mention that we had no data of diabetic status, dyslipidemia and degree of renal dysfunction. These co-morbidities might influence the urinary excretion of lipids in our studied cohort.

Nephrolithiasis patients had increases in oxidative stress and renal tubular damage, and the degree of oxidative stress was correlated with renal tubular damage [20, 21]. Associations of urinary total lipids with markers for oxidative stress and renal tubular injury were found in this study. Using multiple regression analysis, urinary MDA was an

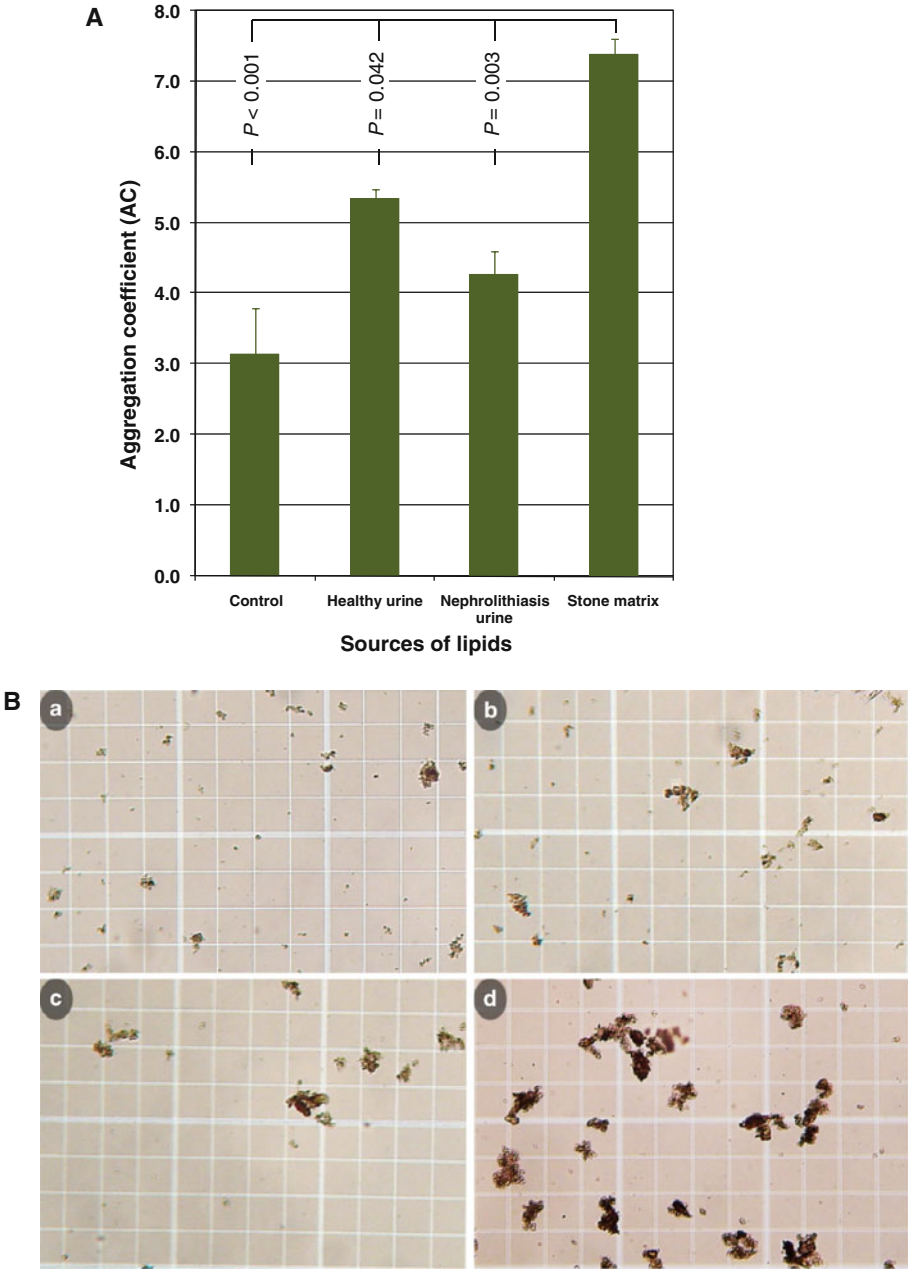

Fig. 6 Effect of total lipids isolated from healthy urines, nephrolithiasis urines and stone matrices on crystal aggregation. **A** Average AC value of stone matrix total lipids was significantly higher than those of urinary total lipids from healthy and nephrolithiasis. **B** Micrographs show crystal aggregates that are corresponded to AC values in **A**: **a** control, **b** healthy urinary total lipids, **c** nephrolithiasis urinary total lipids, **d** stone matrix total lipids. *Error bars* represent standard errors. Magnification ×100

independent predictor for urinary total lipid (data not shown). Our present findings provide a close relationship between increased lipid peroxidation and increased urinary lipid excretion.

Evidences for the presence of lipids in urinary stones, indicated by osmiophilic and sudanophilic substances in stone sections, were initially demonstrated by Khan et al. [9]. In their subsequent study, higher lipid contents in CaOx and calcium phosphate stones than in UA and struvite stones were found [15]. In this study, lipid contents in MAP and CaOx stone matrices were higher than that in UA stone matrix (Fig. 2). Although the exact explanation is not known, the nature of crystals (organic for UA vs. inorganic for CaOx and MAP) might influence the incorporation of lipids into stones.

We clearly demonstrated that lipids extracted from stone matrices promoted formation and aggregation of CaOx crystals, and their capability was significantly higher than lipids extracted from urines of nephrolithiasis and healthy subjects (Figs. 5, 6). Lipids-induced crystal formation was found in dose-dependent manner, and lipids from patients' urine had more efficiency of crystal induction than lipids from healthy urines (Fig. 5). As demonstrated by MOD-TLC, stone matrix lipids contained several classes of lipids including phospholipids/gangliosides, cholesterol, free fatty acids, triacylglycerides, cholesterol esters and unidentified

Table 4 Effect of each lipid class isolated from stone matrices on CaOx crystal aggregation

Purified lipids	Aggregation coefficient (AC)	*P* value
Control	3.13 ± 1.16	
B1 (phospholipids/ gangliosides)	5.73 ± 1.67	0.055
Pooled B2–B5	6.60 ± 0.79	0.004
B6 (cholesterol)	6.07 ± 0.31	0.020
B7 (free fatty acids)	5.73 ± 0.51	0.055
B8 (triacylglyceride)	3.37 ± 0.81	1.000
Pooled B9–B10	6.33 ± 0.32	0.009
B11 (cholesterol esters)	5.40 ± 0.20	0.148

Data presented as mean ± SD. *P* from Bonferroni multiple comparison test versus control

PAS-positive lipids (B2–B5 and B9–B10). The PAS-positive lipids were considered as glycolipids. B2–B5 glycolipids had higher polarity than B9–B10 glycolipids. Glycolipids, cholesterol and free fatty acids were rarely detected in urine samples. These would be the explanation for superior crystal-forming and -aggregating activities of stone matrix lipids compared to the urine lipids. Glycolipids (particularly B5) and cholesterol were more frequently detected in nephrolithiasis urines than in the healthy urines, which may be responsible for a higher crystal-inducing activity of nephrolithiasis urinary lipids than healthy urinary lipids (Fig. 5).

Cholesterol (B6) was significantly capable of inducing crystal formation and aggregation while glycolipids (B2–B5 and B9–B10) were a significant promoter of crystal aggregation. These lipids have some degrees of polarity; they may electrostatically adhere to the crystal surfaces acting as bridges or glue (for glycolipids) in order to cluster the crystals. However, it should be noted that these lipids may be accidentally incorporated into stones as constituents of membrane fragments/vesicles or even the whole cells [9, 10]. We think that urinary cholesterol may play a role in

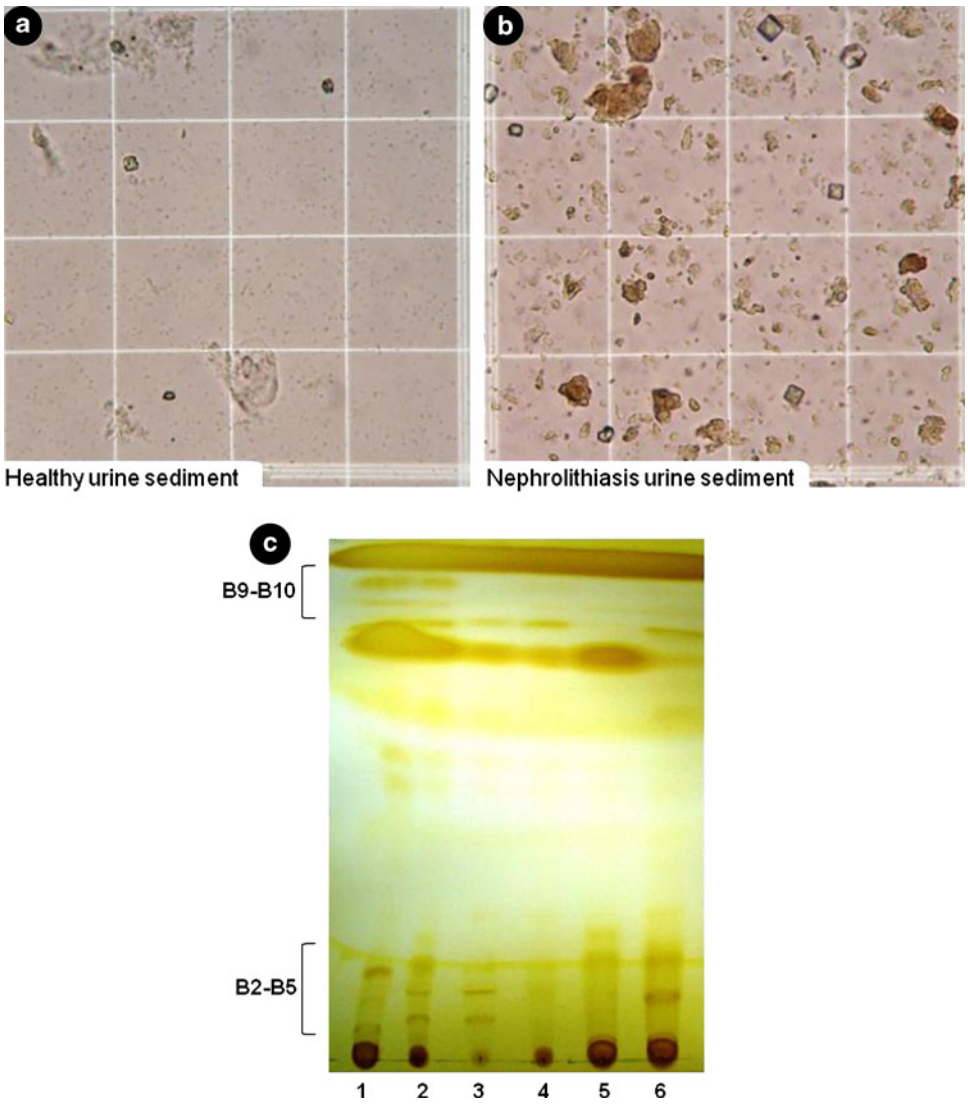

Fig. 7 Urine sediments of healthy and nephrolithiasis subjects and MOD-TLC chromatograms of lipids extracted from urine sediments: **a** micrograph of urine sediment obtained from one of healthy individual, **b** micrograph of urine sediment from nephrolithiasis patient and **c** MOD-TLC chromatographic patterns (iodine staining) of urine sediment lipids from three nephrolithiasis patients (*lanes 1–3*) and three healthy individuals (*lanes 3–6*). Lipids loaded in each lane were normalized by urine volume. More glycolipids (*B2–B5* and *B9–B10*) were found in nephrolithiasis urine sediments compared to healthy urine sediments. Magnification ×200

lithogenesis. Hydroxyl functional group is shown to have small adhesive force on COM surfaces, as measured by atomic force microscopy [22]. In our aggregation system, cholesterol may adhere to crystal surfaces via its hydroxyl moiety rendering the surfaces to be coated with its hydrophobic ring moiety, and these protruding hydrophobic structures form hydrophobic bonds with the nearby cholesterol-coated crystals to form crystal aggregates.

Another interesting finding was that triacylglyceride was an abundant lipid in urines of both nephrolithiasis patients and healthy subjects, but it was scanty in stone matrices. Moreover, it had no effect on crystal formation and aggregation. This may suggest that triacylglyceride, as neutral lipid, has a lesser role in lithogenesis compared to the other polar lipids. Its incorporation into stone matrix might be accidental or passive process. Study of Khan et al. [10] showed that lipids in urine selectively incorporated into crystal matrices depended on their polarity. CaOx crystals induced in healthy urine incorporated, respectively, about 6, 18 and 100% of neutral lipids, glycolipids and phospholipids that presented in the urine.

In conclusion, level of urinary lipids in nephrolithiasis patients was associated with extents of oxidative stress and renal tubular injury. Triacylglyceride was abundant in urines, but scarcely incorporated into stones. Glycolipids and high-polar lipids were principal lipid constituents in human kidney stone matrix and functioned as lithogenic promoters. Cholesterol purified from stone matrices acted as both crystal nucleator and aggregator.

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