Original article

Urinary oxalate excretion is increased in calcium oxalate nephrolithiasis patients and associated with increased urinary capacity of calcium oxalate crystallization

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Background: Calcium oxalate (CaOx) stone is the most common type of stones formed in the urinary tract. Formation of CaOx stone is driven by increased CaOx crystallization in urine.
Objective: To develop a new test, called indole-reacted calcium oxalate crystallization index (iCOCI), and to measure the capability of urine to produce CaOx crystals.
Methods: One hundred samples of 24-h human urine samples obtained from CaOx stone-forming subjects (SFS, n = 50) and non-stone subjects (NSS, n = 50). The levels of oxalate were determined by two methods, i.e., oxalate oxidase method and iCOCI test. Unpaired student *t* - test, Pearson's correlation, and receiver operating characteristic (ROC) analysis were performed.
Results: The urinary oxalate levels in CaOx SFS were significantly higher than those in NSS. Likewise, urinary iCOCI levels in SFS were significantly greater than those in NSS. ROC analysis revealed the area under ROC curves of 0.588 (95% CI: 0.472 - 0.703) and 0.897 (95% CI: 0.838 - 0.956) for urinary oxalate and iCOCI tests, respectively. At the cutoff of 6.2 mg/day, sensitivity and specificity of urinary oxalate test were 60.0% and 58.0%, respectively. The urinary iCOCI test yielded the sensitivity of 88.0% and specificity of 74.0% at the cutoff of 0.8 COM eqv., g/day. Urinary oxalate was positively correlated with urinary iCOCI both in NSS and SFS groups, but it was more pronounced in SFS group.
Conclusion: The urinary oxalate and iCOCI levels in patients with CaOx nephrolithiasis were increased compared to individuals without kidney stones. Diagnostic performance of urinary iCOCI test was remarkedly greater than the urinary oxalate test. Increased urinary oxalate was highly correlated with increased urinary iCOCI. Plausibly, increased urinary oxalate contributed to increased capability of urine to form CaOx crystals.

Keywords: Calcium oxalate, crystallization, iCOCI test, kidney stone, urinary oxalate, urolithiasis.

Nephrolithiasis is commonly found worldwide, but it is more prevalent in the tropical countries, including Thailand.[1] The most common type of kidney stones is calcium oxalate (CaOx). Etiology of stone disease is complex and multifactorial.[2, 3] Low fluid intake, high oxalate intake, and low consumption of citrus fruits are the main dietary risk factors for CaOx stone formation.[2, 4, 5] Increased urinary oxalate excretion is one of the well-known conditions that predisposes to CaOx stone formation.[6] Normal range of urinary oxalate excretion in urine is 20 - 40 mg/day, and urinary oxalate level higher the normal range is called hyperoxaluria.[7] Urinary oxalate concentration largely depended on dietary consumption of oxalate.[4, 5, 8, 9] Therefore, dietary oxalate restriction is a recommended preventive measure to reduce the risk of stone formation and recurrence. Oxalate intake < 100 mg/day is advised to decrease the predisposition of stone recurrence.[10] It is well recognized that increased urinary oxalate level can increase risk of CaOx supersaturation and crystallization more than increased urinary calcium level.[4] Furthermore, evidence suggests that even mild or moderate hyperoxaluria can be a cause of recurrent CaOx stone formation.[6, 11, 12] Therefore, measurements of urinary oxalate and capability of urine to crystalize CaOx are essential to estimate the risk of CaOx stone development.

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We recently established a new test to measure the urinary potential of CaOx crystallization, called indole-reacted calcium oxalate crystallization index (iCOCI), and showed that urine dilution and urine oxalate concentration are the key determinants of urinary CaOx crystallization.[13, 14] However, association of urinary oxalate with CaOx crystallization capacity have not been investigated in CaOx stone patients. In this study, we measured urinary oxalate and CaOx crystallization capacity (by iCOCI test) in CaOx stone patients compared with the non-stone individuals and determined whether urinary oxalate level was associated with urinary iCOCI level.

Materials and methods

CaOx nephrolithiasis patients and 24-h urine specimens

Human urine specimens used in this study were the same set of samples that we used in the previous study.[13] The 24-hour urine samples were collected from the healthy volunteers (non-stone subjects, NSS, n = 50) and kidney stone patients (CaOx stone-forming subjects, SFS, n = 50) admitted to Mahasarakham province during 2017 and 2018. The inclusion criteria for NSS group were both males and females aged 18 years and over, and for SFS group were patients with kidney stones aged 18 years and over who were admitted to Mahasarakham Hospital for stone treatment. For exclusion criteria of NSS group, subjects with urinary tract infections or having a history of kidney stone were excluded. In SFS group, patients with hematuria, using drugs that caused urine color change or having urinary tract infection or any malignancies were excluded. The research protocol was reviewed and approved by the Institutional Ethics Committees, Faculty of Medicine, Chulalongkorn University (no. IRB 419-62).

Urinary oxalate determination by oxalate oxidase method

Levels of soluble oxalate in urine sample was measured by the oxalate oxidase (OxOx) enzymatic method. The OxOx enzyme was purchased from the Roche diagnostic, Germany, and horseradish peroxidase (HRP) was purchased from the Sigma-Aldrich, USA. The urine sample was pretreated by activated charcoal (Sigma-Aldrich, USA) (80 mg of charcoal mixed with 1 mL of the urine sample) by incubating at room temperature for 10 min with interval mixing. Treated urine sample was centrifuged at 10,000 xg for 5 min, and supernatant was collected. First, the supernatant (20 µL) was placed into 6 separated wells (in 96-well plate): 2 wells for sample blank, 2 wells for sample test, and 2 wells for oxalate spike (internal standard). Second, type I water (5 µL) was added to sample blank wells and sample test wells, and 5 µL of 1.25 mM sodium oxalate was added to the oxalate spiked wells. Third, the reaction reagent containing 110 µL of working 3, 32 , 5, 52 - Tetramethylbenzidine (TMB) chromogen substrate (0.1 mg/mL) in succinate buffer pH 3.8, 40 µL of 2 mg/ml OxOx enzyme, and 1 µL of 2 mg/ml HRP enzyme. The reagent mix for the sample blank (without OxOx) was prepared by mixing 110 µL of working TMB in succinate buffer pH 3.8, 40 µL of distilled water, and 1 µL of 2 mg/ml HRP enzyme. The reaction was performed in 96-well plate and incubated at room temperature for 10 min in the dark. The absorbance at 650 nm optical density (OD) was measured using a microplate reader (Thermo Scientific, USA). Urinary oxalate was calculated according to the formula as shown below; 250 µM is the final concentration of the spiked oxalate and 1.25 is the dilution factor.

$$\text{Urinary oxalate } (\mu M) = \frac{(\text{OD sample test - OD sample blank})}{(\text{OD oxalate spike - OD sample test})} \times 250 \times 1.25$$

$$\text{Urinary oxalate (mg/day)} = \frac{\text{urinary oxalate } (\mu M)}{1000} \times$$

$$88.02 \text{ (molecular weight of oxalate)} \times \text{Urine volume (24 hour, L)}$$

Urinary iCOCI test

The iCOCI measurement was performed according to our previous report.[13] Urine samples were centrifuged at 4,000 rpm for 5 min. Supernatant was collected and filtered through 0.22 μm membrane for iCOCI testing. Various concentrations of calcium oxalate monohydrate (COM) solution were prepared in 2 N HCl for creating COM standard curve. The filtered urine sample was spiked with oxalate to achieve final concentration of 2 mM oxalate. Oxalic acid solution (2 mM final concentration) was prepared to be used for subtraction of absorbance from the oxalate-spiked urine. $CaCl_2$ solution was added to the oxalate-spiked urine and the oxalic acid solution and then incubated at 37°C for 10 min. The generated crystals were harvested by entrifugation and redissolved in 2 N HCl for the indole reaction. Indole reagent (1 mg/mL) was freshly prepared in concentrated H_2SO_4. The crystal solution was mixed 1:1 with indole reagent. The mixture was incubated at 80°C for 45 min. Absorbance at 530 nm was measured. The iCOCI value was calculated as fully described in our previous study.[13]

Statistical analysis

Data were presented as mean ± standard deviation (SD). Differences in urinary iCOCI levels between NSS and SFS were carried out by unpaired student *t* - test. Association of two measurements was assessed by the Pearson's correlation test. Receiver operating characteristic (ROC) analysis was performed to assess how well the urinary oxalate and iCOCI tests were able to distinguish SFS from NSS. Cutoff values were chosen based on the highest accuracy of the tests. GraphPad Prism 9.0 was used for all statistical calculations. *P* - value < 0.05 was considered as statistically significant.

Results

Urinary levels of oxalate and iCOCI increased in patients with CaOx nephrolithiasis

Characteristics of the studied cohorts are shown in Table 1. Mean age of the CaOx SFS were higher than the NSS, and the SFS group had more men than in NSS. The 24-h urine volume between these two groups was not statistically different.

Level of urinary oxalate in CaOx SFS (11.1 ± 11.9 mg/day) was significantly higher than in NSS (6.4 ± 3.0 mg/day) ($P = 0.0075$) (Figure 1A). For urinary iCOCI measurement, level of urinary iCOCI in CaOx SFS (2.1 ± 2.8 COM eqv., g/day) was also significantly higher than that in NSS (0.6 ± 0.3 COM eqv., g/day) ($P = 0.004$) (Figure 1B).

Diagnostic performance of urinary oxalate and urinary iCOCI measurements

ROC analysis was performed to evaluate how good the urinary oxalate and urinary iCOCI tests be able to separate the CaOx stone subjects from individuals without stones. The result showed that the area under ROC curve (AUC) of urinary oxalate was of 0.588 (95% CI: 0.472 – 0.703) ($P = 0.131$) (Figure 2A) and indicated that urinary oxalate test had no discriminatory power to separate CaOx SFS from NSS. In contrast, the AUC of urinary iCOCI test was of 0.897 (95% CI: 0.838 – 0.956) $P < 0.001$) (Figure 2B). This finding indicated that urinary iCOCI test had an excellent performance to discriminate CaOx SFS from NSS. The cutoff values of urinary oxalate and urinary iCOCI tests were selected based on the highest accuracy. At the cutoff of 6.2 mg/day, urinary oxalate test provided sensitivity and specificity of 60.0% and 58.0%, respectively. For the urinary iCOCI test, it provided the sensitivity of 88.0% and specificity of 74.0% at the cutoff of 0.8 COM eqv., g/day.

Table 1. Characteristics of the studied subjects.

Characteristics	NSS	CaOx SFS	*P* - values
Number of subjects (n)	50	50	
Gender (male: female)	16:34	34:16	
Age (years)	47.5 ± 8.8	54.8 ± 10.8	0.0004
Urine volume (mL)	1352 ± 598	1513 ± 487	0.1413

NSS: Non-stone subjects, CaOx SFS: Calcium oxalate stone-forming subjects

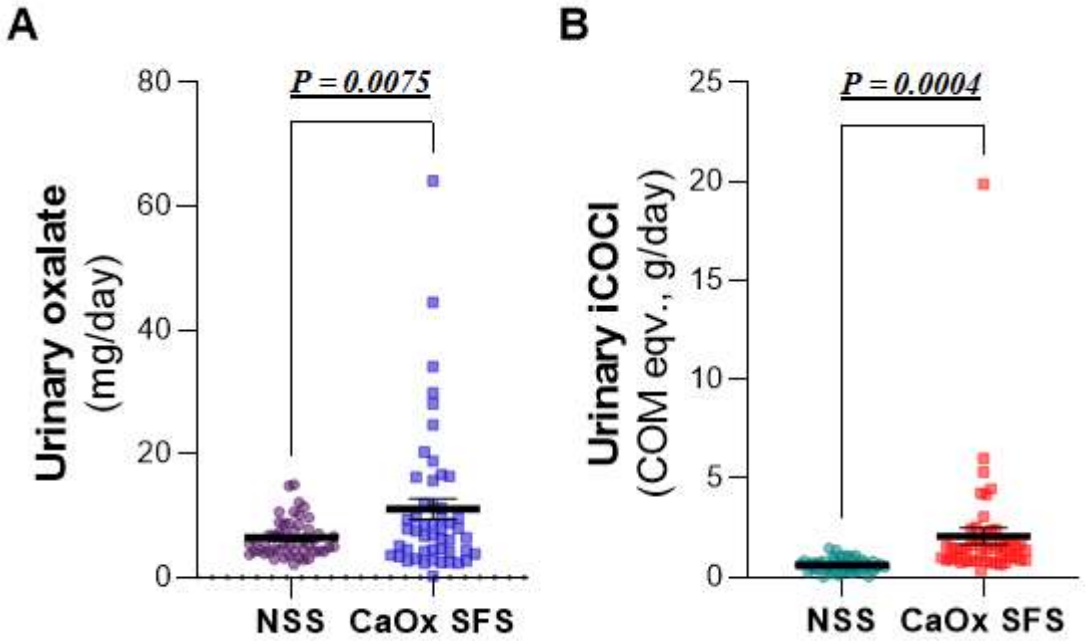

Figure 1. Levels of urinary oxalate and iCOCI compared between NSS and CaOx SFS. **(A)** Urinary oxalate level in CaOx SFS was significantly higher than in NSS. **(B)** Urinary iCOCI values of CaOx SFS was also significantly higher than that of NSS. Error bars indicate mean ± Standard error of the mean (SEM).

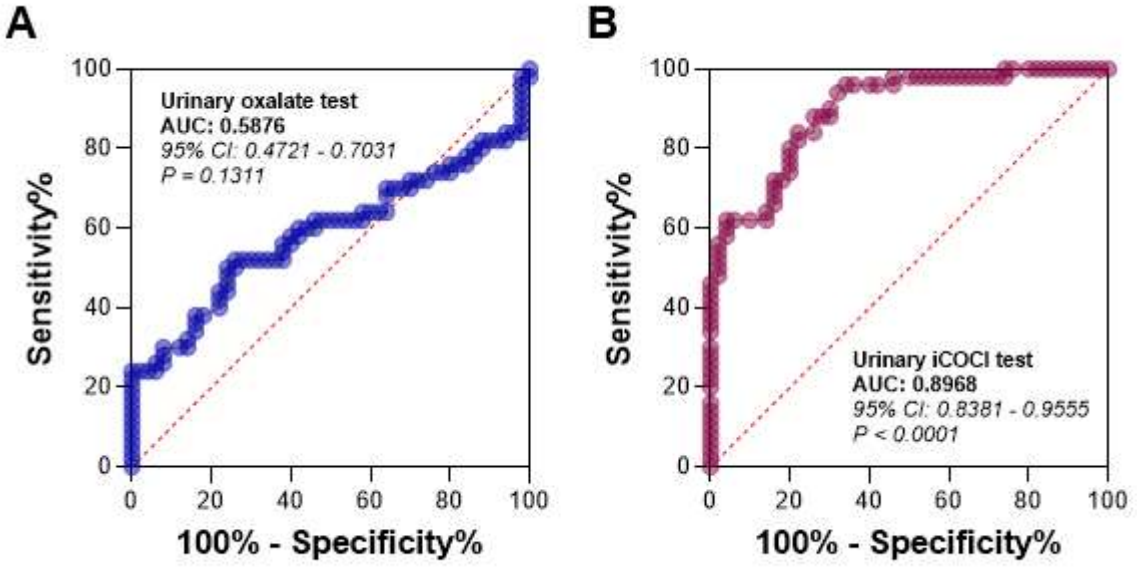

Figure 2. ROC curves of **(A)** urinary oxalate and **(B)** urinary iCOCI measurements. The area under ROC curve (AUC) of urinary oxalate was of 0.588 (95% CI: 0.472 – 0.703), indicating no discriminatory ability to diagnose patients with CaOx stone. The AUC of urinary iCOCI test was of 0.897 (95% CI: 0.838 – 0.956), indicating an excellent diagnostic performance for CaOx nephrolithiasis.

Urinary iCOCI level strongly correlated with urinary oxalate in the CaOx SFS

The iCOCI test fundamentally measures the total capacity of urine sample to form CaOx crystals under the supersaturated condition, and it has an excellent performance to diagnose CaOx nephrolithiasis.[13] Whether urinary iCOCI level was associated with urinary oxalate was evaluated in this study. In all subjects (n = 100), urinary iCOCI linearly correlated with urinary oxalate (0.796, $P < 0.001$). In NSS group, there was a positive correlation between urinary iCOCI and urinary oxalate ($r = 0.541$, $P < 0.001$). In CaOx SFS, a more pronounced positive correlation between urinary iCOCI level and urinary oxalate level was observed ($r = 0.792$, $P < 0.001$) (Figure 3).

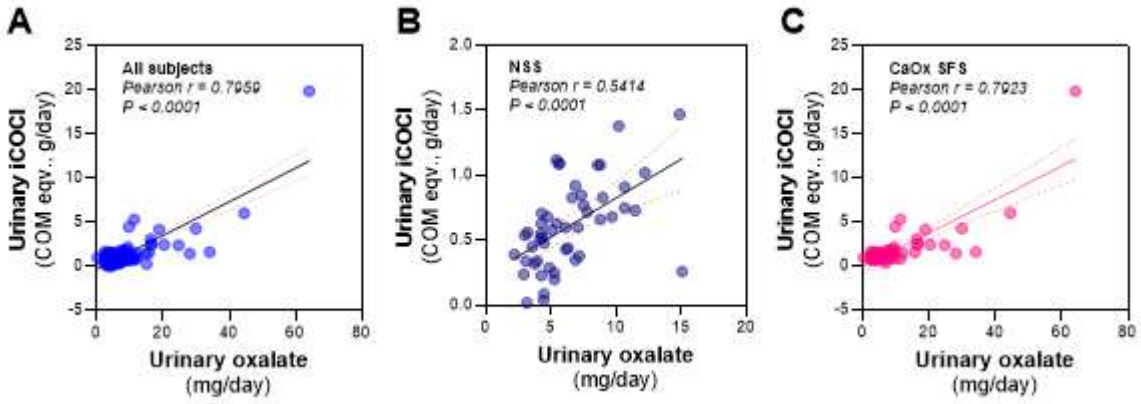

Figure 3. Correlation between urinary iCOCI and urinary oxalate levels. **(A)** In all subjects, urinary iCOCI significantly correlated with urinary oxalate ($r = 0.796$, $P < 0.001$). **(B)** In NSS group, urinary iCOCI was also positively correlated with urinary oxalate ($r = 0.541$, $P < 0.001$). **(C)** In CaOx SFS, the more pronounced positive correlation between urinary iCOCI and urinary oxalate was observed ($r = 0.792$, $P < 0.001$).

Discussion

CaOx stone formation is initially driven by supersaturation of calcium and oxalate ions that consequently lead to increased CaOx crystallization.[15, 16] Increased water load and decreased urine oxalate concentration effectively reduce the urinary supersaturation and hence reducing the CaOx crystallization.[15] The saturation of urinary salts has been measured by several methods, but the most commonly used method is EQUIL2.[17] We recently developed the new method, called iCOCI method, to measure the urine capacity of CaOx crystallization based on the induction of urine supersaturation of CaOx.[13, 14] We demonstrated *in vitro* that urine dilution and urine oxalate concentration were the key contributors of CaOx crystal formation.[14] In this study, we explored further in human subjects to determine if urinary CaOx crystallization (measured by iCOCI method) was correlated well with the urinary oxalate level. Our data clearly show that urinary iCOCI strongly correlated with urinary oxalate, especially in the SFS group. The levels of urinary oxalate and urinary iCOCI were relatively low in the NSS group, and some of the values were even close to zero. Basically, quantitative measurement of substances at a very low level or at a level close or below the limit of detection of the test trends to be inaccurate and imprecise. This might be the reason why the correlation between urinary oxalate and urinary iCOCI levels in CaOx SFS group was stronger than in the NSS group. Although the current finding was only the association study, it suggested that increased urinary oxalate might be a cause for increased urinary iCOCI level. Further experimental study is required to warrant this speculation.

In this study, we clearly demonstrated that patients with CaOx stones had increased urinary oxalate and urinary iCOCI relative to the subjects without stone formation. The finding was not novel, but we confirmed that urinary oxalate and CaOx crystallization were important factors to measure to estimate the risk for CaOx stone formation and recurrence. To our knowledge, the first study that demonstrated an increased urinary excretion of oxalic acid in nephrolithiasis patients was reported by Hodgkinson A.[18] Determination of urinary oxalate by oxalate oxidase enzymatic method was formally introduced by Buttery JE, *et al*.[19] Now, it is well recognized that excreted amount of oxalate in urine principally contributes to CaOx stone formation.[20]

Based on the present ROC result [21], urinary iCOCI test was recommended to perform as the routine measurement because it provided an excellent diagnostic power to distinguish CaOx stone formers from healthy individuals. According to the literature, normal range of urinary oxalate excretion is less than 45 mg/day, and it is normally used for the Western patients.[22] In our subjects, urinary oxalate levels in both CaOx SFS and NSS were still in the normal range but the average level of urinary oxalate in the SFS group was about two times higher than the NSS group. This might suggest that the urinary oxalate excretion between the Western and Asian subjects were different. Further studies are required for verify this speculation.

We established our oxalate oxidase method for urinary oxalate determination based on the previous published procedure.[23] Pretreatment of urine samples with charcoal was an essential step to remove interfering substances that could not be omitted.[24] We also evaluated the diagnostic performance of our urinary oxalate determination. We found that the area under ROC curve of urinary oxalate test was not significantly greater than 0.5, indicating that the test was no better than the random guessing. We concluded that urinary oxalate level was significantly increased in CaOx stone patients, but it alone could not be used as a good diagnostic marker for CaOx nephrolithiasis. Dussol B, *et al*. showed that the sensitivity and specificity of urinary oxalate (mmol/day) for classifying calcium stone patients were 44.0% and 65.0%, respectively.[25] Rossi MA, *et al*. also reported low sensitivity (59.0% in men, 36.0% in women) and specificity (42.0% in men, 79.0% in women) of 24-hour urine oxalate level for detecting supersaturation of CaOx.[26] Therefore, our present findings corroborated well with the previous evidence.

Limitations of the study should be mentioned. Sample size of SFS and NSS groups were relatively small. There were men in SFS group more than in NSS group. The SFS were significantly older than the NSS. These would limit the extrapolation of the present findings.

Conclusion

We demonstrated that levels of urinary oxalate and iCOCI in patients with CaOx nephrolithiasis were significantly increased compared with those without stone formation. Urinary iCOCI test had an excellent diagnostic power to separate CaOx SFS from NSS, but urinary oxalate test had no discrimination. Increased urinary oxalate was highly correlated with increased urinary iCOCI, particularly in SFS group. Although our finding was only an association study, it was likely that increased urinary oxalate contributed to increased capability of urinary CaOx crystallization.

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Conflicts of interest statement

Each of the authors has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

The present review is based on the references cited. Further details, opinions, and interpretation are available from the corresponding authors on reasonable request.

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