Relationship between Components of urinary crystallites and Formation of Calcium Oxalate Stones---An Investigation of 275 Cases of Patients with Calcium Oxalate Stones

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Abstract-[Purpose] This study aimed to investigate the components of urinary crystallites in 275 cases of patients with calcium oxalate (CaOx) stones compared with the components of stones. [Methods] X-ray diffraction (XRD), Fourier-transform infrared (FT-IR) spectrometer, selected area electron diffraction (SAED) and fast Fourier transformation (FFT) of high-resolution transmission electron microscopy (HRTEM) were performed. [Results] The main components of urinary crystallites in patients with CaOx stones were calcium oxalate monohydrate (COM), uric acid (UA), calcium oxalate dihydrate (COD) and calcium phosphate (CaP), which were similar to the components of stones (COM, COD, UA, and CaP). [Conclusions] The formation of CaOx stone was related to the presence of COM, UA, and CaP crystals in urine. UA and CaP crystals could induce the development of CaOx stones by heterogeneous nucleation. The formation mechanism and the influential factors of CaOx stones were discussed on the basis of the components of urinary crystallites.

Key words: Urine crystallite; urinary stones; calcium oxalate; XRD; TEM

I. INTRODUCTION

Urolithiasis is a frequently occurring urologic disease worldwide [1, 2]. For example, the overall probabilities of forming stones in Saudi Arabia, North America, and Europe are 20%, 13%, and 5% to 9%, respectively [3]. Urinary stone is mainly treated by extracorporeal shock wave lithotripsy (ESWL) [4]. However, stone recurrence and regrowth rates after ESWL are high, particularly reported as 40% in 3 years, 74% in 10 years, and 98% in 25 years after therapy [3].

Urinary stones can be classified as acidic (e.g., UA and cystine), alkaline (e.g., MAP), and neutral (e.g., CaOx) stones based on their chemical properties. Urinary stones with different components vary not only in their physical properties (such as hardness, morphology, and solubility) but also in their chemical properties (phase component, acid-base property, and element). Therefore, the curative effects of drug and extracorporeal shock wave lithotripsy (ESWL) are quite different. However, the same methods and the same drugs are currently used in clinical practice to treat patients with stones, and the specific compositions of the stones are not distinguished. Hence, different curative effects and low treatment rates are obtained. If the specific type of urinary stones can be determined before stones are removed and treatment, a personalized treatment can be used, thereby preventin the recurrence of urinary stones.

There exists a close relation between urinary crystallites and kidney stones [5-7]. The disappearance of a specific type of urinary crystallites (e.g. cystine, struvite) in human urine may reduce calculi recurrence trend of corresponding crystal types (e.g. cystine stone, struvite calculus), whereas their constant occurrence or re-occurrence generally indicates the calculi activity or relapse, respectively. Therefore, it is likely to shed light on clinically suiting remedy to the case and personalized treatments by analyzing the urine crystalline components.

Based on the discussed above, the components of urinary crystallites in 275 cases of patients with calcium oxalate (CaOx) stones were investigated and compared with the components of stones.

II. MATERIALS AND METHODS

A. Reagents and instruments

Absolute ethanol was of analytical purity. Glass vessels were cleaned with distilled water.

X-ray diffraction (XRD) results were recorded using a D/max 2400 X-ray diffractometer (Rigaku, Tokyo, Japan) with Ni-filtered Cu Ka radiation (k = 1.54 A°) at a

scanning rate of 2° min^{-1} (40 kV, 30 mA). The divergence and scattering slit was 1° for the range of 5° < 2θ< 60°. A Nicolet 6700 Fourier-transform infrared (FT-IR) spectrometer (Nicolet Company, USA) was also used. TEM was conducted on a HRTEM (JOEL 2100F) with a maximum acceleration voltage of 200 kV and lattice resolution of 0.19. To determine the component of urinary crystallites, we performed HRTEM, fast Fourier transformation (FFT), and selected area electron diffraction (SAED) of HRTEM. FFT analysis in the Digital Micrograph software was also conducted to obtain the patterns.

B. Collection and component detection of stones

275 cases of calcium oxalate stones were collected from patients with calculi after surgery. The age of patients ranged from 20 years to 80 years with a mean age of 46.3 years. Urinary stones were then disinfected with 75% alcohol to kill bacteria, cleaned with distilled water, and placed in a dust-free incubator at 45 °C to dry. Afterward, these urinary stones were ground to powder by using an agate mortar for XRD and FT-IR characterization.

C. Collection, treatment, and XRD and FT-IR detection of urinary crystallites

Fasting morning urines samples were collected from patients who were instructed to fast before sampling was performed. pH was detected and 2% NaN_3 solution (10 mL/L urine sample) was added to the urine samples as antiseptic. Approximately 30 mL of urine was then centrifuged at 4000 r/m for 15 min, and the supernatant was removed. The residual was washed and allowed to stand for 3 min; the supernatant was then removed. This process was repeated twice. The residual was placed on clean glass slides by using a dropper. The slides were dried in a vacuum desiccator for further XRD and FT-IR characterization.

D. HRTEM detection of urinary crystallites

In addition to XRD and FT-IR characterization, HRTEM, SEAD and FFT were performed to investigate the components and elements found in urinary crystallites. The urine sample was initially subjected to ultrasound treatment for 5 min. Approximately 5 μL of urine was submerged in a copper mesh by using a microsyringe; urine was preliminarily dried using an absorbent paper from the back of the mesh to remove the water content of urine. After treatment was performed, most of the soluble salts (such as NaCl and urea) in urine were removed by the filter paper. The mesh was then stored in a desiccator for 2 d before HRTEM analyses were performed.

III. RESULTS AND DISCUSSION

A. Component analysis of stones

The components of 452 cases of urinary stones were analyzed by XRD and FT-IR; among these cases, 275 showed CaOx stones (pure CaOx stones and mixed stones with CaOx as the main component).

Fig. 1 showed the XRD spectra of the stones from 12 cases of representative patients with CaOx stones. We detected the peaks at d = 5.93, 3.65, 2.97, 2.49, 2.35, 2.26, 2.07 and 1.98 Å, which were assigned to ($\bar{1}$01), (020), ($\bar{2}$02), (112), (130), (202), (321) and ($\bar{3}$03) planes of calcium oxalate monohydrate (COM) crystal [8], respectively. We also detected the peaks at d =6.55, 3.86, 3.18, 3.08, and 1.79 Å, which were assigned to (200), ($\bar{2}$11), (121), (221), and (223) planes of uric acid (UA), respectively. We detected the peaks at d =2.26, 2.07 1.89, 1.74 and 1.69 Å, which were assigned to (1016), (0018), (238), (330), and (508) planes of calcium phosphate (CaP), respectively, and the peaks at d =3.44, 2.78, 1.94 and 1.84 Å, which were assigned to (002), (112), (222) and (213) planes of hydroxyapatite (HAP), respectively. That is, the main components of CaOx stones were found to be COM, UA and CaP.

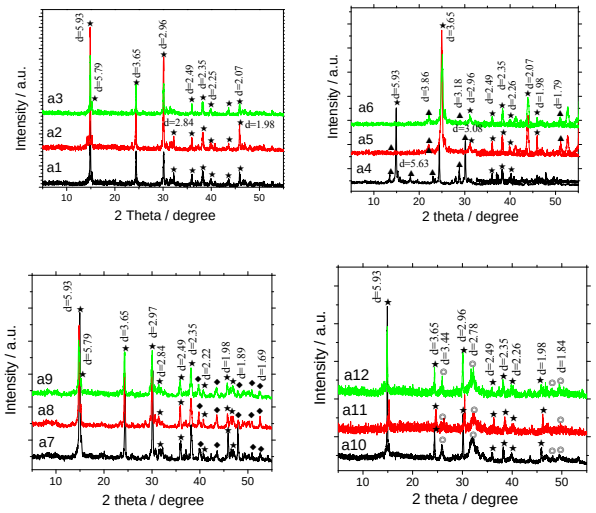

Figure 1. XRD patterns of representative 12 cases of CaOx stones. (★: COM; ▲: uric acid; ◆: β-$Ca_3(PO_4)_2$; ◎: hydroxyapatite)

Fig. 2 showed the FT-IR spectra of representative 12 cases of CaOx stones. The peaks of coordinated water were located at 3488 cm^{-1} to 3219 cm^{-1}. These broad peaks were attributed to the symmetrical stretching vibration and asymmetrical stretching vibration of OH- of coordinated water molecules. The main antisymmetric carbonyl stretching band ($\nu_{as}(COO^-)$) specific to the oxalate family occurs at 1622cm^{-1} for COM. The secondary carbonyl stretching bands, the metal-carboxylate stretch, $\nu_s(COO^-)$ is located at 1317 cm^{-1} for COM. The absorption peaks at 2924, 1383, and 1023 cm^{-1} were assigned to CaP. The peaks at 3017, 1673, 1123, and 993 cm^{-1} were assigned to UA. FT-IR detection results were consistent with those obtained from XRD.

B. Component analysis of urinary crystallites in patients of CaOx stones

XRD and FT-IR were performed to investigate the components of urinary crystallites of the 275 cases of CaOx stone formers above. Some of the samples were further investigated by HRTEM and FFT to accurately study their components. The results are listed as follows.

Urinary crystallites were detected in 171 cases of patients among the 275 cases of patients with CaOx stone as the main component (Fig. 3); these crystallites mainly contained COM, UA, and COD and a small amount of CaP and calcium hydrophosphate (Table 1).

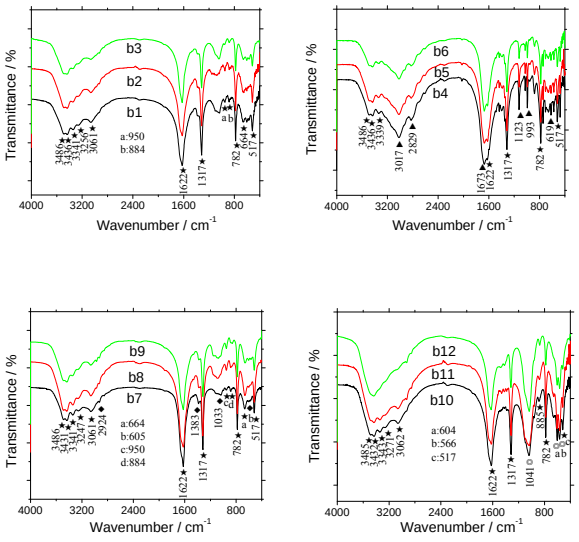

Figure 2. FT-IR spectra of representative 12 cases of CaOx stones.

TABLE I. COMPONENTS OF URINARY CRYSTALLITES IN 275 CASES OF CAOX STONE PATIENTS

No.	Components of Urinary crystallites	Number	Percentage / %
1	COM+UA *;**	79	28.7
2	COM+UA+CaP	19	6.9
3	COM+COD +UA+CaP	7	2.5
4	COD+CaP	3	1.1
5	CaP+COM	3	1.1
6	COM+COD+UA	4	1.5
7	COD+CaP+ $CaHPO_4$+COM	3	1.1
8	UA	53	19.3
9	No crystallites	104	37.8
Total		275	100

[*] COM: calcium oxalate monohydrate; UA: uric acid; COD: calcium oxalate dihydrate; CaP: calcium phosphate; $CaHPO_4$: calcium hydrogen phosphate.
[**]The components of urinary crystallites were sorted in terms of their contents (from high to low)

1) XRD analysis of urinary crystallites

Fig. 4 showed the XRD spectra of urinary crystallites from 8 cases of the representative patients with CaOx stones above. We detected the peaks at d = 5.93, 3.65, 2.97, 2.49, 2.36, 1.98 and 1.73Å, which were assigned to ($\bar{1}01$), (020), ($\bar{2}02$), (112), (130), ($\bar{3}03$) and ($\bar{3}23$) planes of COM, respectively [8]. We also detected the peaks at d =4.91, 3.93, 3.16 and 2.93 Å, which were assigned to (210), ($\bar{2}11$), ($\bar{1}21$) and ($\bar{2}21$) planes of uric acid (UA) crystal, respectively, and d =8.17, 2.60 and 2.20 Å, which were assigned to (1012), (220) and (404) plane of CaP.

That is, the main components of CaOx stones were found to be COM, UA and CaP, and urinary crystallites were also composed of COM, UA and CaP.

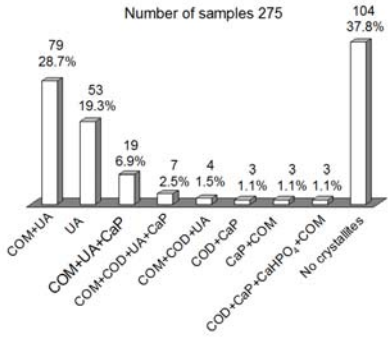

Figure 3. Components of urinary crystallites in 275 cases of CaOx stone patients

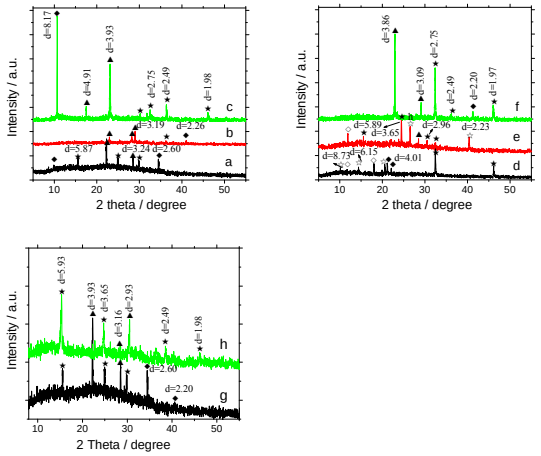

Figure 4. XRD spectra of urinary crystallites in 8 representative CaOx stone patients. Symbols: ★: COM; ☆: COD; ▲: uric acid; ◇: ammonium urate; ◆: $Ca_3(PO_4)_2$.

2) HRTEM analysis of urinary crystallites

Fig. 5 showed the images obtained from HRTEM and FFT of the urinary crystallites.

The sub-maximal diffraction peaks of COM (d = 5.76, 2.96, 2.75, 2.49, 2.35 and 2.07 Å) were detected and was assigned to the ($\bar{1}10$), ($\bar{2}02$), (420), (112), (130) and (321) plane of COM crystal, respectively [8].That is, the main crystal plane of COM were detected in these HRTEM images.

3) SAED analysis of urinary nanocrystallites

Selected area electron diffraction (SAED) was carried out to further characterize the components of urinary nanocrystallites. Fig. 6 showed the images of (SAED of the urinary crystallites. We simultaneously detected the presence of COM, UA and CaP. In SAED pattern, we detected an interplanar spacing of d = 3.08 Å, which was assigned to the strongest plane ($\bar{1}21$) of UA.
We also detected an interplanar spacings of d = 2.60 and 1.41 Å, which were assigned to the (220) and (1514) planes of ß-CaP, respectively. Interplanar spacings of d = 2.49, 1.98 and 1.72 Å, which were assigned to (112), ($\bar{3}23$), and ($\bar{3}03$) planes of COM crystal [8], respectively.

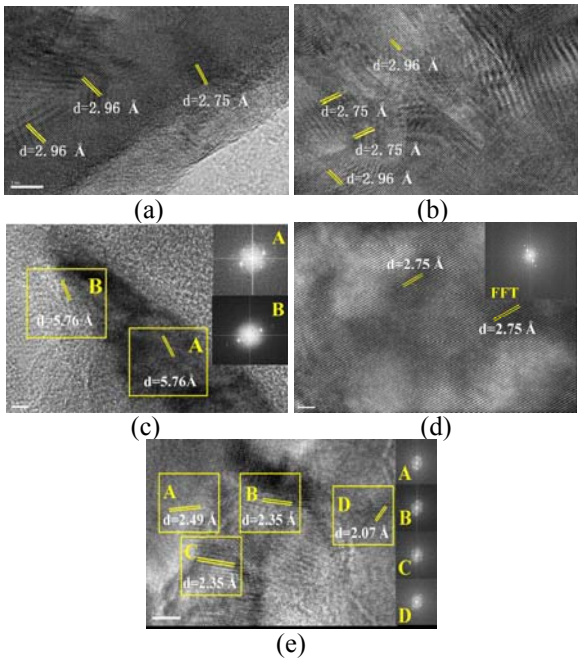

Figure 5. FFT images of HRTEM of urinary crystallites in CaOx stone patients.

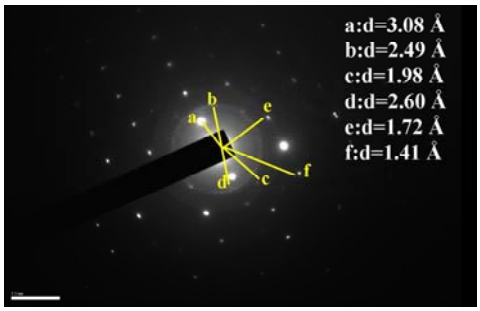

Figure 6. SAED images in different areas of urinary crystallites in patients with CaOx stones.

IV. DISCUSSION

XRD, FT-IR, HRTEM, and FFT were performed to investigate the components of urinary crystallites. The results showed that the main components of urinary crystallites in patients with CaOx stones were UA, COM, and CaP (Table 1), which are similar to the components of stones (COM, COD, and small amounts of UA, CaP, and HAP) (Fig. 1). The results also indicated that the components of urinary crystallites highly indicated the activities of stone-related diseases.

CaOx is the most common component of urinary stones and often accompanied by other components. This result is attributed to CaOx crystals that can be formed regardless of urine pH. In acidic urine, CaOx usually forms stones accompanied by uric acid; in alkaline urine, CaOx usually forms stones accompanied by HAP, CaP, and MAP [9].

In a previous study [10], the components of CaOx stone in its interior region are CaOx and CaP; in the surrounding region, CaOx stone contains CaOx, indicating that CaP crystals are nidus to induce CaOx stone formation. In urine with pH > 6, all kinds of crystallites, such as CaP, ACP [11], HAP [12], and carbonate apatite [13], can be formed. These crystallites can function as nidus to induce the formation of CaOx stone and then produce mixed stones accompanied by phosphate [14].

The formation of CaOx stone is often related to hyperuricosuria. In urine with pH < 5.5, the solubility of UA significantly decreases and the precipitated UA crystals can function in heterogeneous nucleation to induce the formation of CaOx stone; as a result, COM/UA stone is produced [15].

It could be seen from Fig. 4 that UA and CaP were detected. These results provided evidence that UA and calcium phosphate nanocrystallites act as nidus to induce COM development/growth, leading to stone formation.

It was reported that the presence of UA crystals could induce the development of COM through heterogeneous nucleation [16]. UA can serve as a nidus to induce the generation, precipitation, and aggregation of CaOx crystals [17]. In addition, UA crystals, which are better than mucoproteins (a glycoprotein) and cell debris as a nucleating agent, can induce the development of COM as a heterogeneous nucleating agent [18]. Therefore, CaOx stone formation is closely related with hyperuricosuria [19].

On the other hand, various kinds of phosphates are present in urine, such as CaP [20], amorphous calcium phosphate (ACP) [21], hydroxyapatite [22], carbapatite, and so on. All of them can act as nidus to induce the development of COM calculi.

V. CONCLUSION

XRD, FT-IR, and HRTEM were performed to study the components of urinary crystallites in 275 cases of patients with CaOx stones. The main components of urinary crystallites in patients with CaOx stones were calcium oxalate monohydrate (COM), uric acid (UA), calcium oxalate dihydrate (COD) and calcium phosphate (CaP), which were similar to the components of stones (COM, COD, UA, and CaP). The results showed that the formation of the CaOx stones was closely related to the components of urinary crystallites.

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